



Finding cures. Saving children.

Pitfalls of sc/snRNA-seq

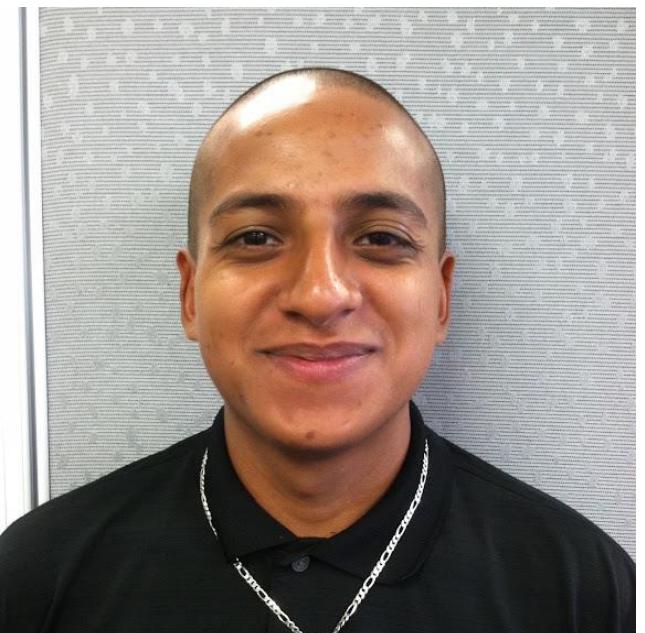
October 30, 2025

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Pitfalls of sc/snRNA-seq workshop outline

- Purpose: highlight analysis steps where pitfalls occur
- Explore, fine tune, especially if data returns unexpected results
- Going to cover:
 - Cell Level QC and Filtering
 - Normalization, Variable Feature Selection, Scaling, Regression
 - Dimensionality Reduction, Clustering, Visualization
 - Integration
 - Differential Expression Analysis
 - Cell Type Annotation
- Focused on analysis in Seurat, but pitfalls are general to any sc/snRNAseq analysis



Pre-Seurat QC Steps

- Look at quality of raw sequencing with fastqs
 - Run FastQC, review sequencing metrics
- Look at quality of alignment
 - Review mapping quality, number of cells, genes, etc.
- See DNB Bioinformatics Core trainings for additional info



[Intro to sc/snRNA-seq workshop](#)

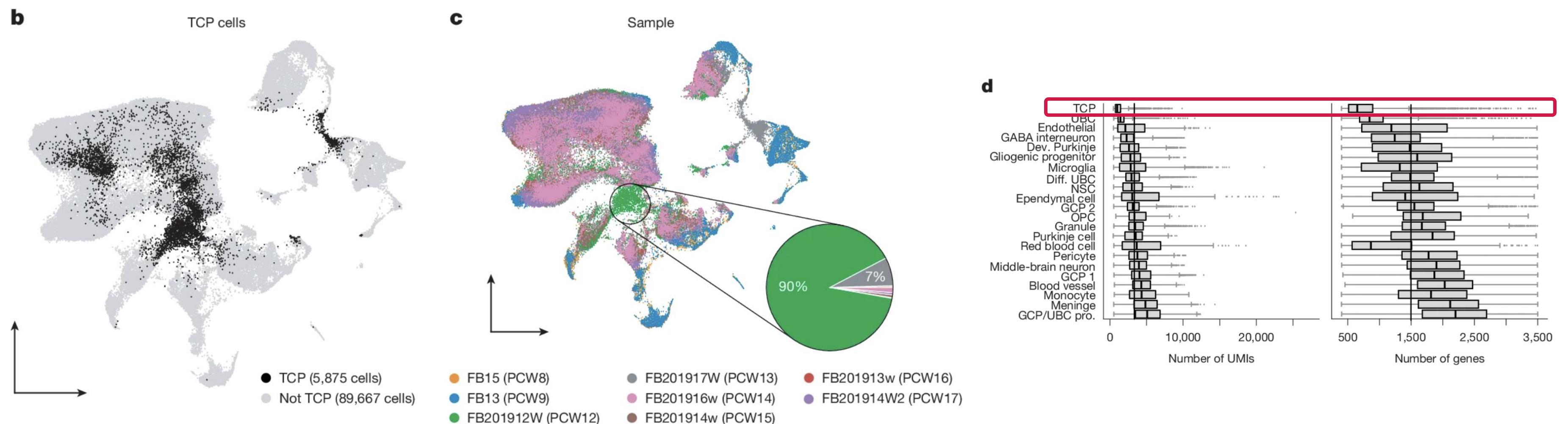


Cell Level QC and Filtering

- First step after alignment, always filter cells
- Low quality cells not informative of true biology
- Skews results, interpretation, can lead to false conclusions
- Cutoffs depend on specific project, data type, sequencing depth
- Choosing appropriate cutoffs is important
 - Too low, keep low quality cells
 - Too high, lose informative cells



Cell Level QC and Filtering



[Smith \(2025\). Nature.](#)

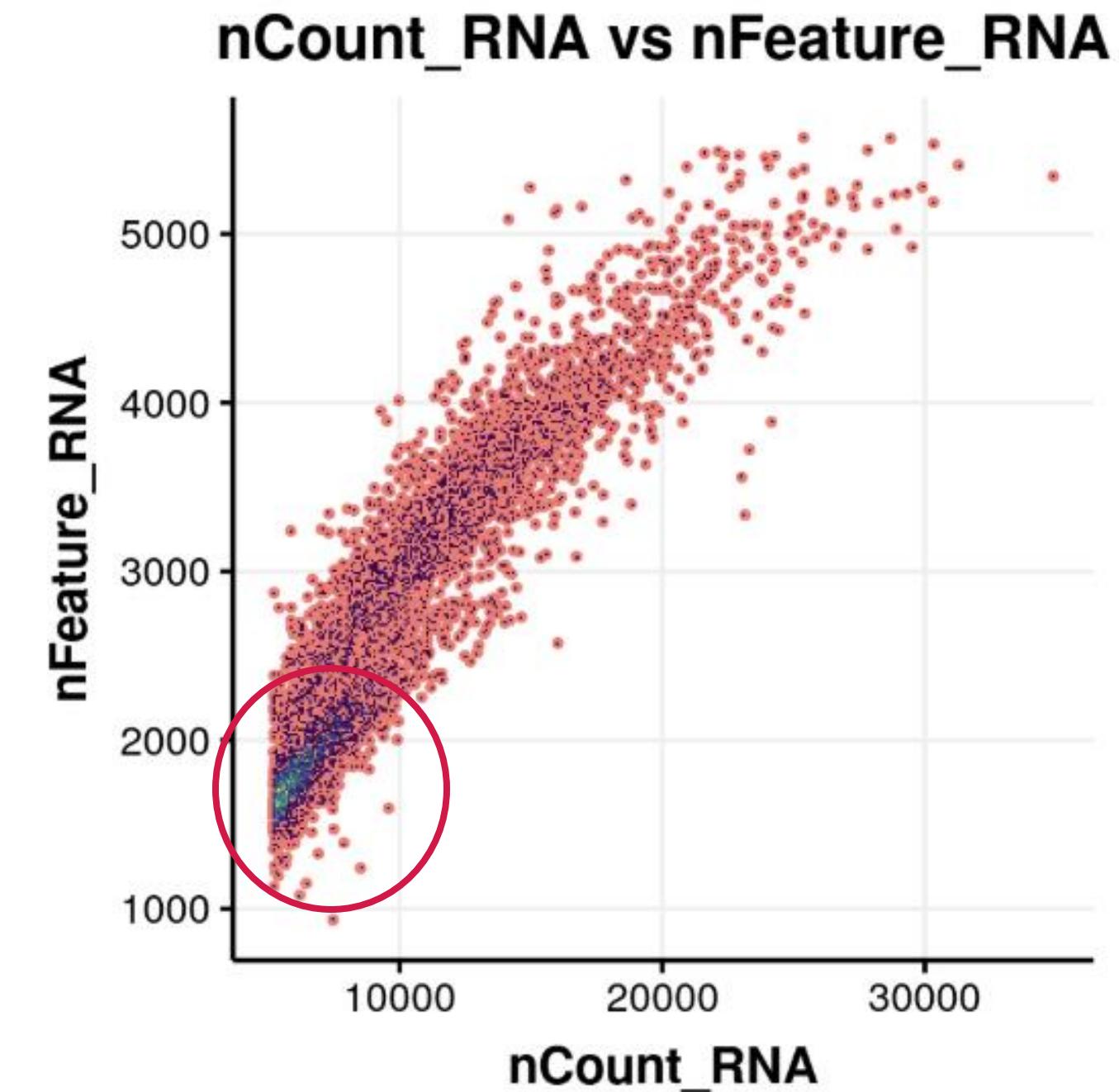
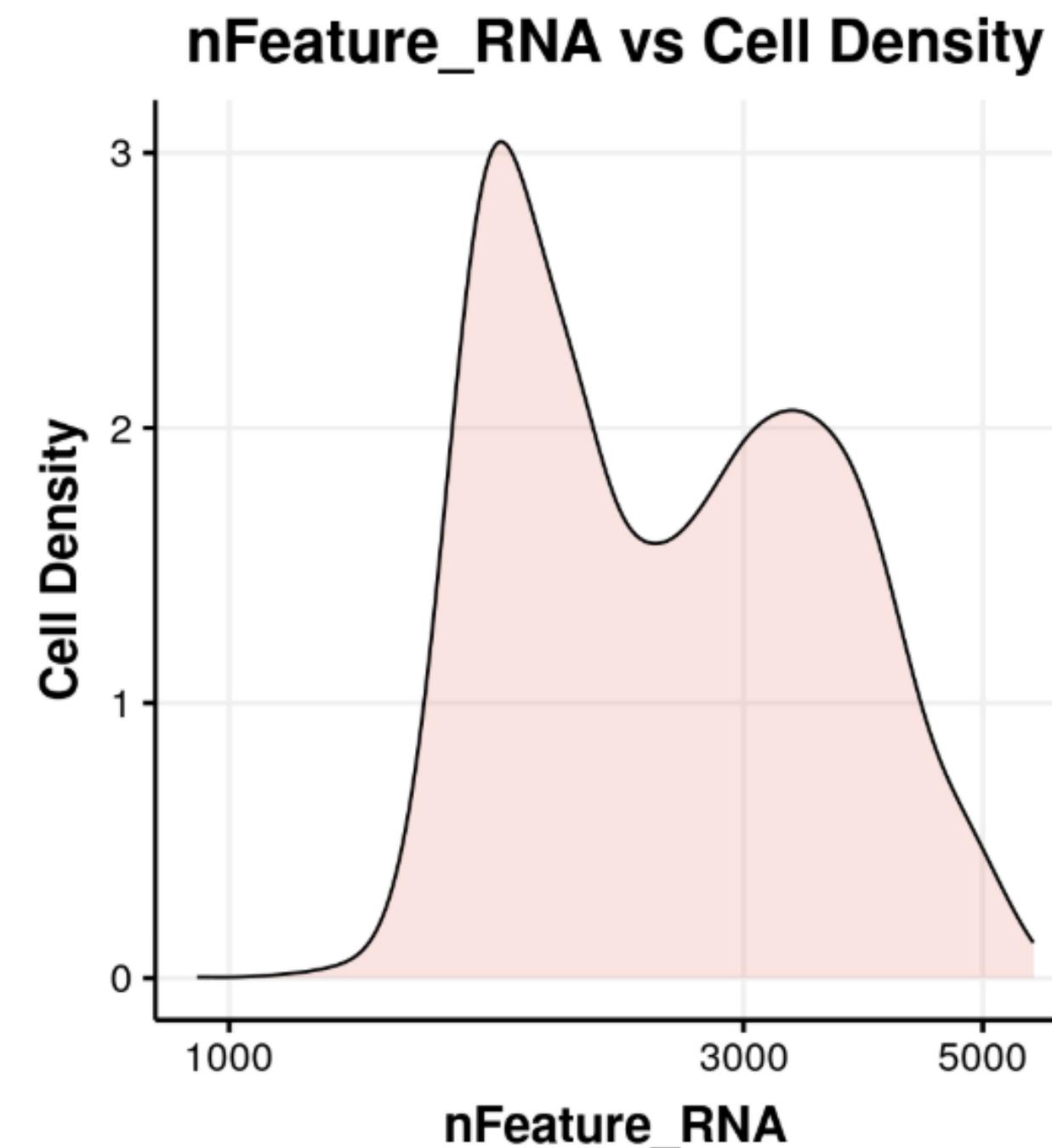


Cell Level QC and Filtering

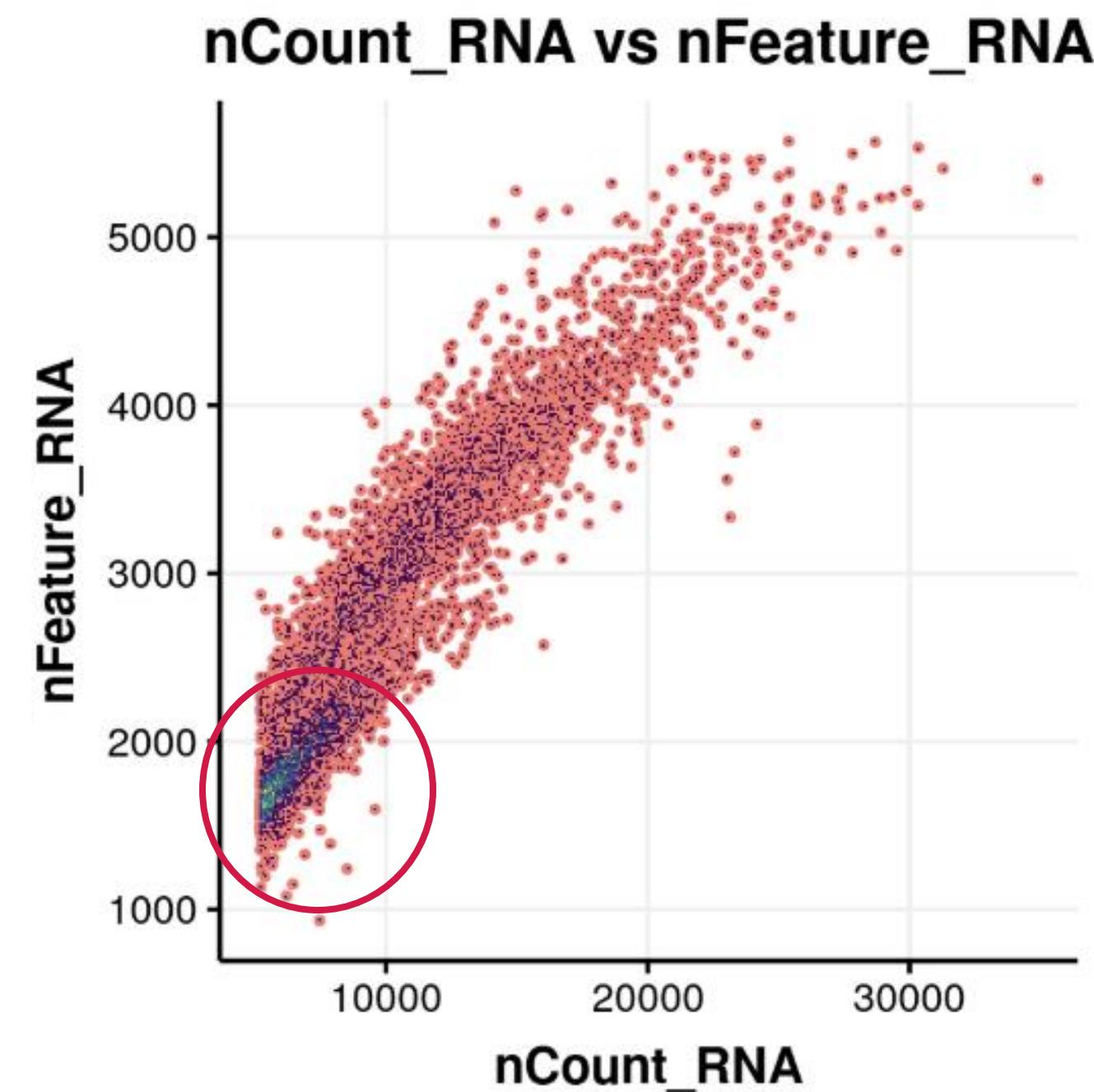
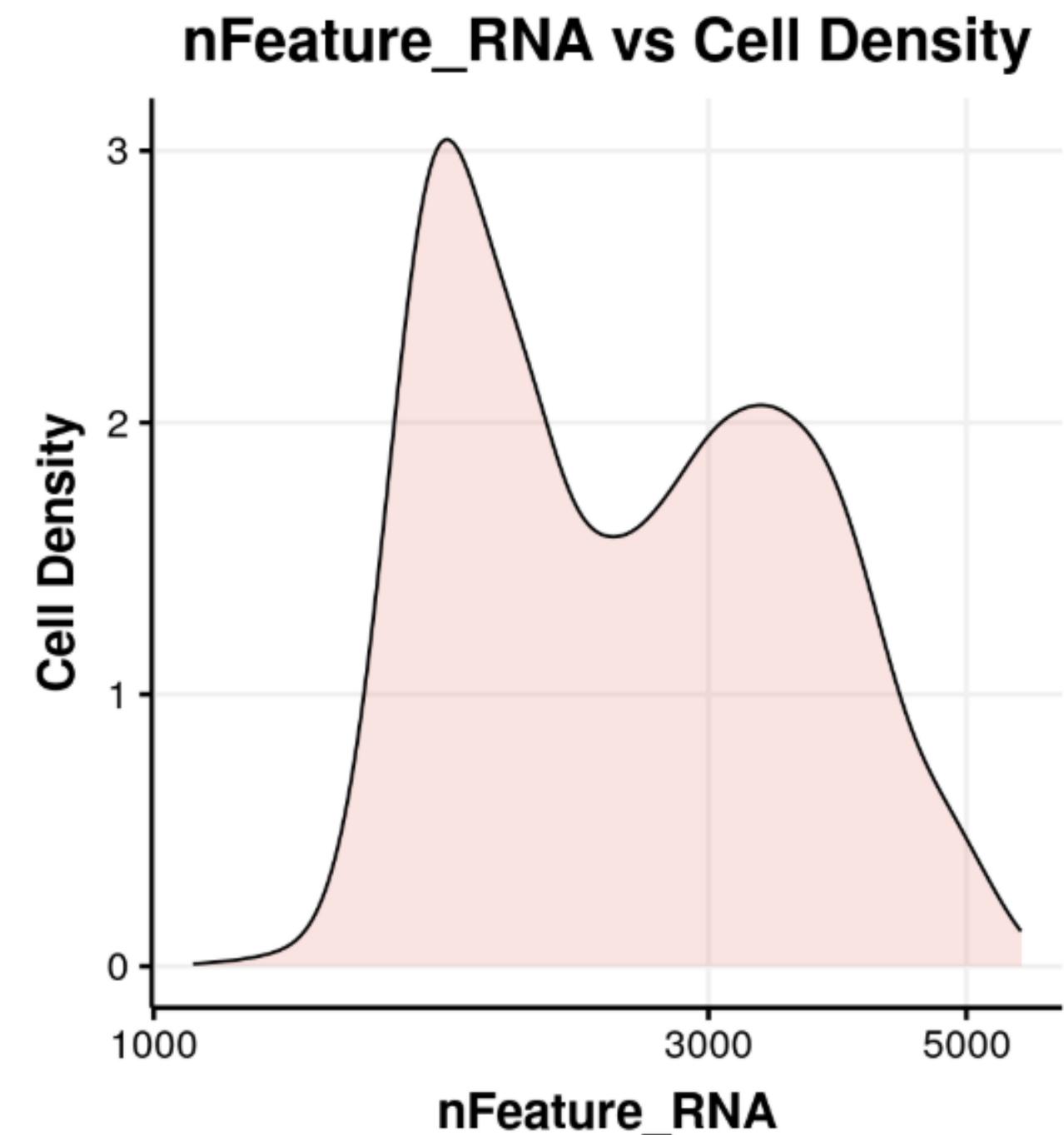
- Minimum: at least 300 genes, at least 500 UMI, less than 5% (scRNA-seq) or 1% (snRNA-seq)
mitochondrial expression
- Consider applying outlier filters, e.g. median absolute deviation (MAD)
- Apply filtering to samples individually
- Test multiple parameter sets
- Search literature for cutoffs used for similar cell types, models
- Look at distributions, other plots of metrics
 - Bimodal distributions, indications of lower diversity, cells grouping on filtering metrics in UMAP



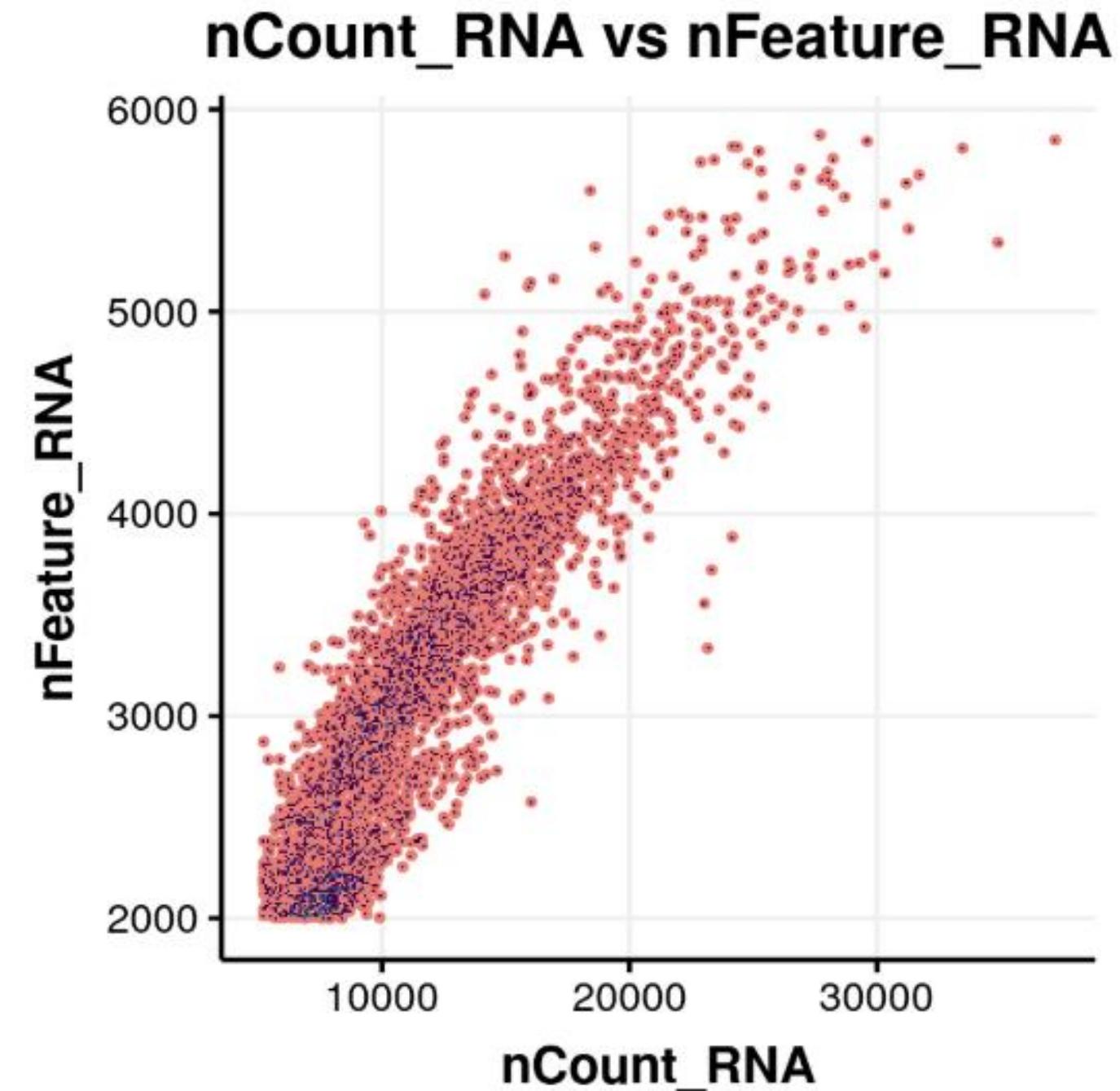
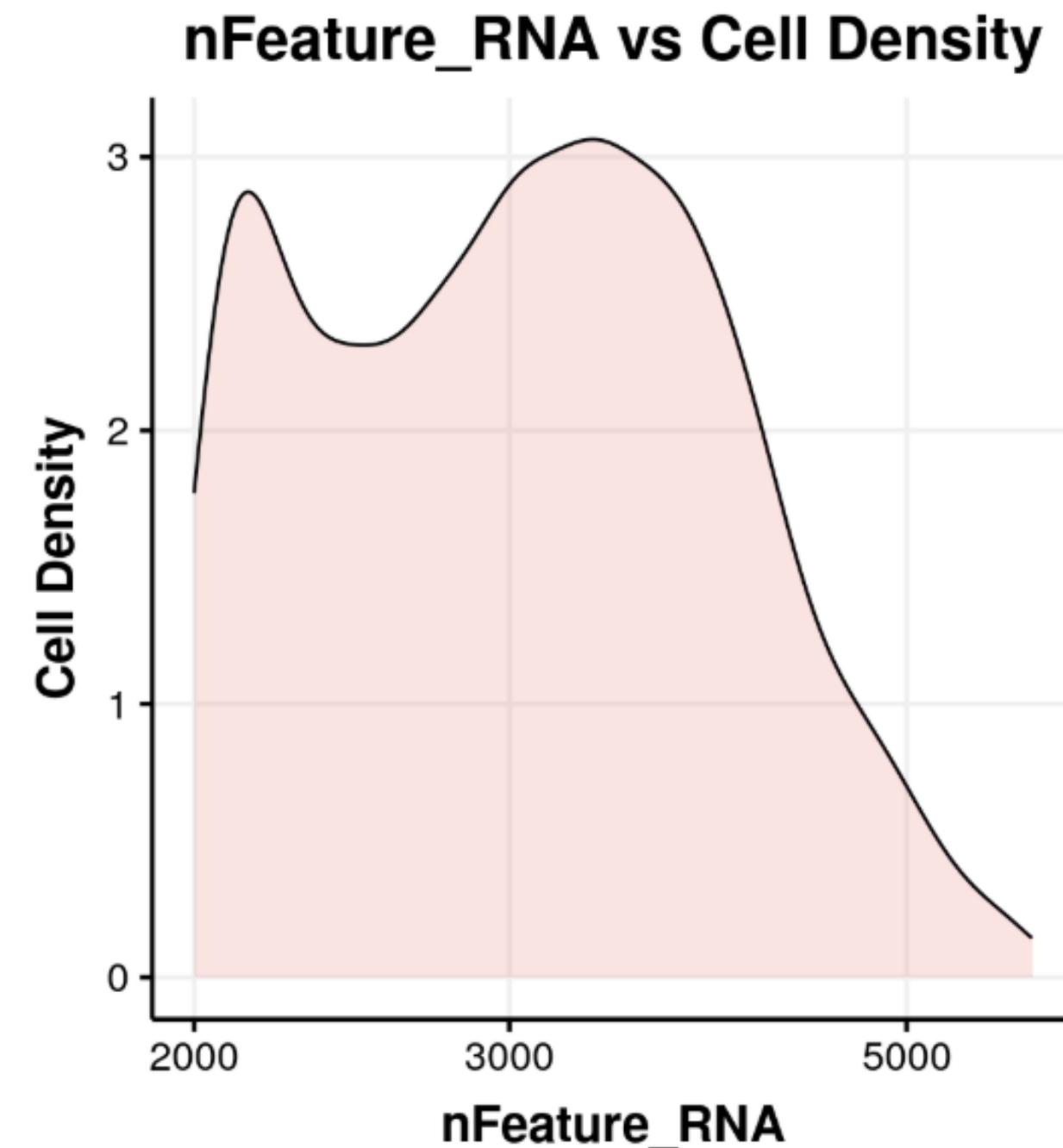
Example: 300 gene + 500 UMI, n cells = 4,837



1k gene + 2k UMI, n cells = 4,836



2k gene + 3k UMI, n cells = 3,279

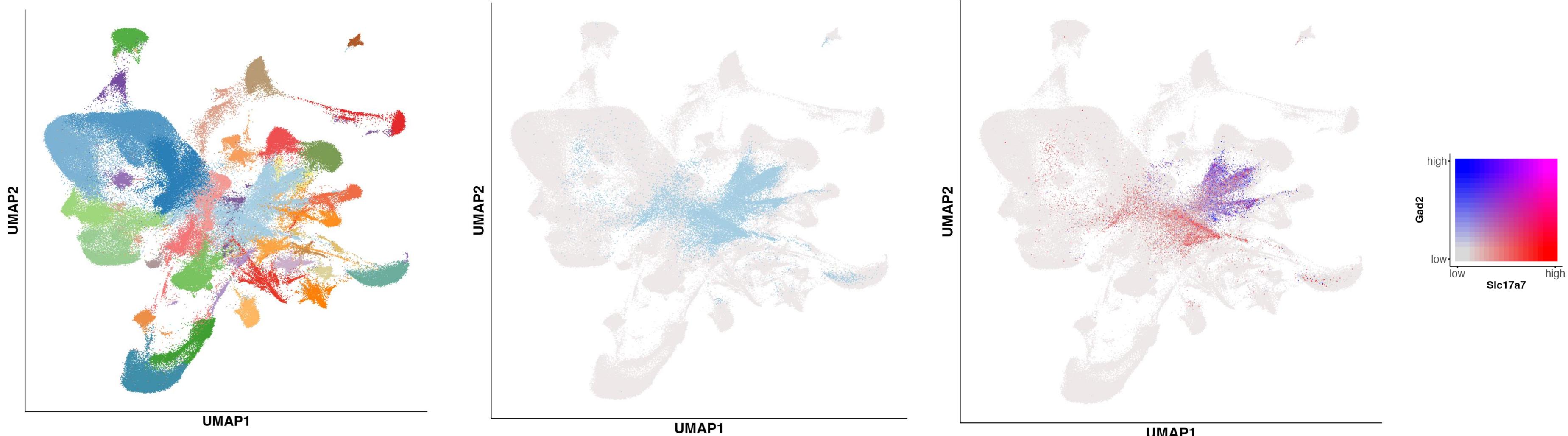


Cell Level QC and Filtering

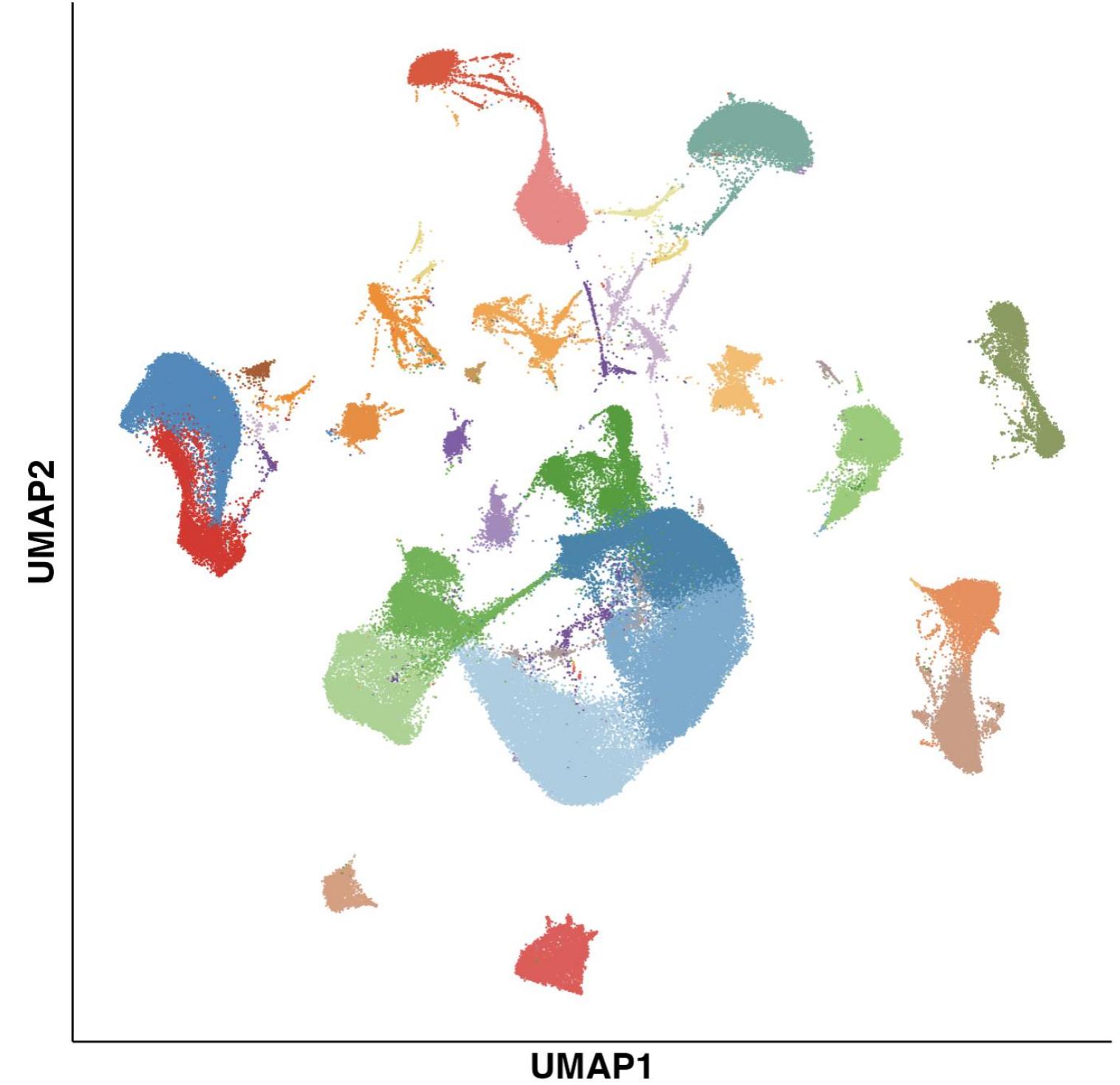
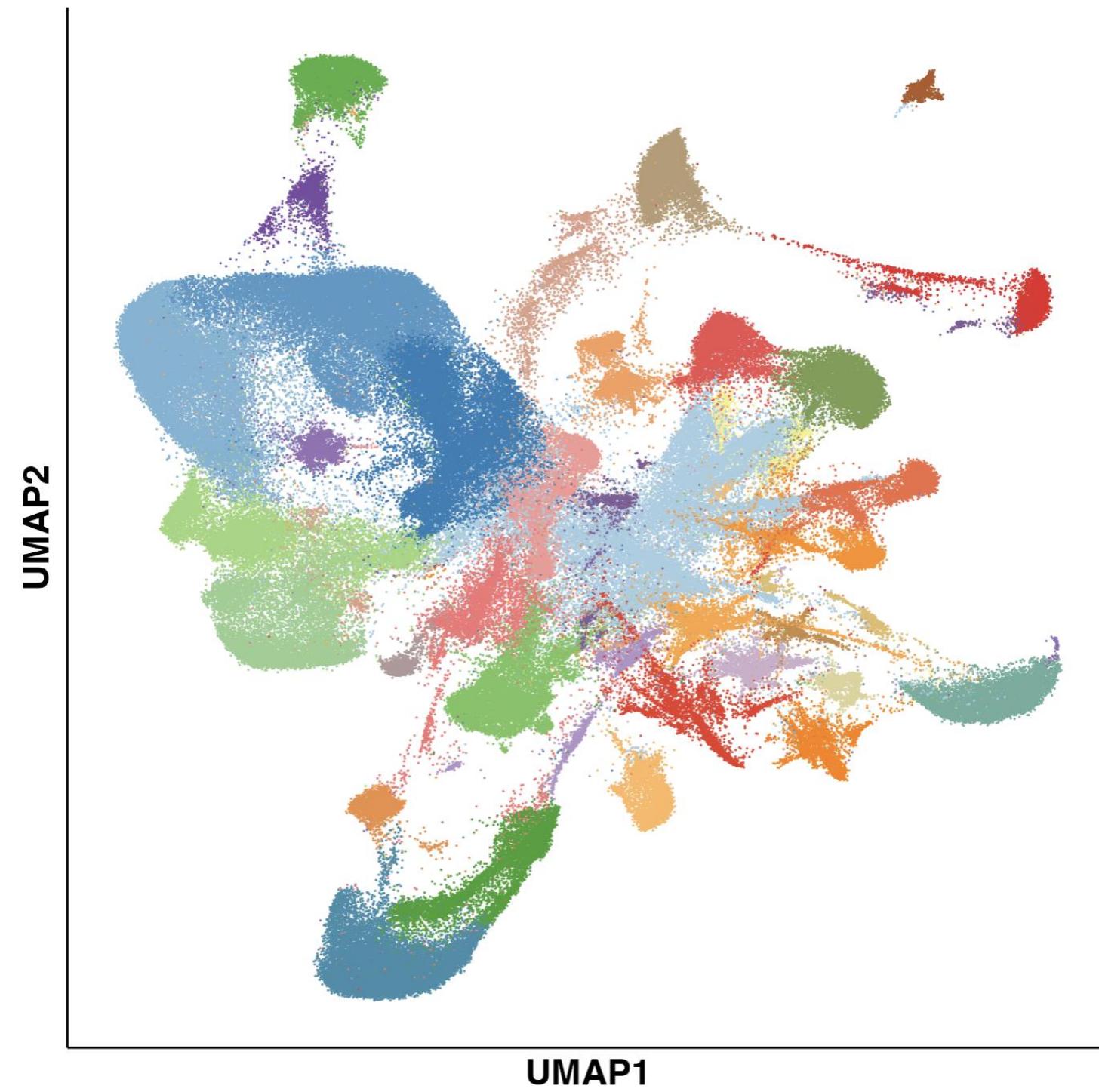
- May need to correct for ambient RNA contamination
- May need to filter out doublets
- Do not recommend correcting, filtering initially
- Tools can struggle with differentiating cell types, overlapping expression
- Patterns in UMAPs, expression could indicate contamination, doublets
 - Spiderwebbing (ambient RNA contamination)
 - Lack of distinct clusters
 - Overlap in mutually exclusive marker expression



Cell Level QC and Filtering

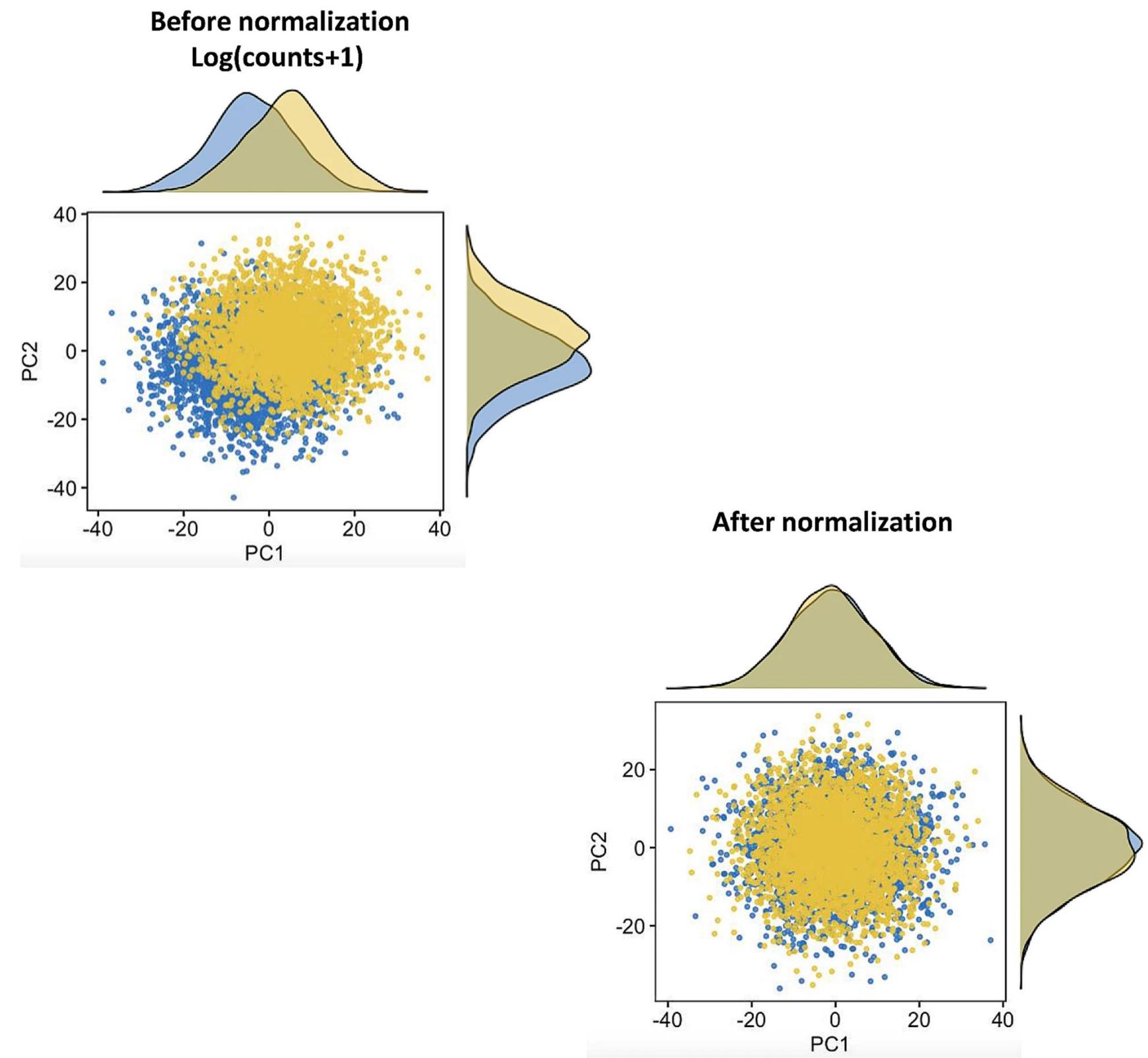


Cell Level QC and Filtering



Normalization

- Important to make gene counts comparable
 - Relevant within and across samples
 - Reduces sources of technical noise
- Seurat has several methods:
 - NormalizeData()
 - LogNormalize (default)
 - Relative Counts
 - Center Log Ratio Transformation
 - SCTransform()
- Must apply the same method across samples



[Cuevas-Diaz Duran \(2024\). BMC Genomics.](#)



Highly Variable Gene (HVG) Selection

- Identify genes highly expressed in some cells, lowly expressed in others
- HVGs impact downstream results
 - Used for principal component analysis (PCA)
 - PCs used for clustering, non-linear dimensionality reduction, etc.
- Focus on HVGs shown to highlight biological signals
- Feature selection method is important
 - Variance alone may not reflect biology
 - Consider mean-variance relationship



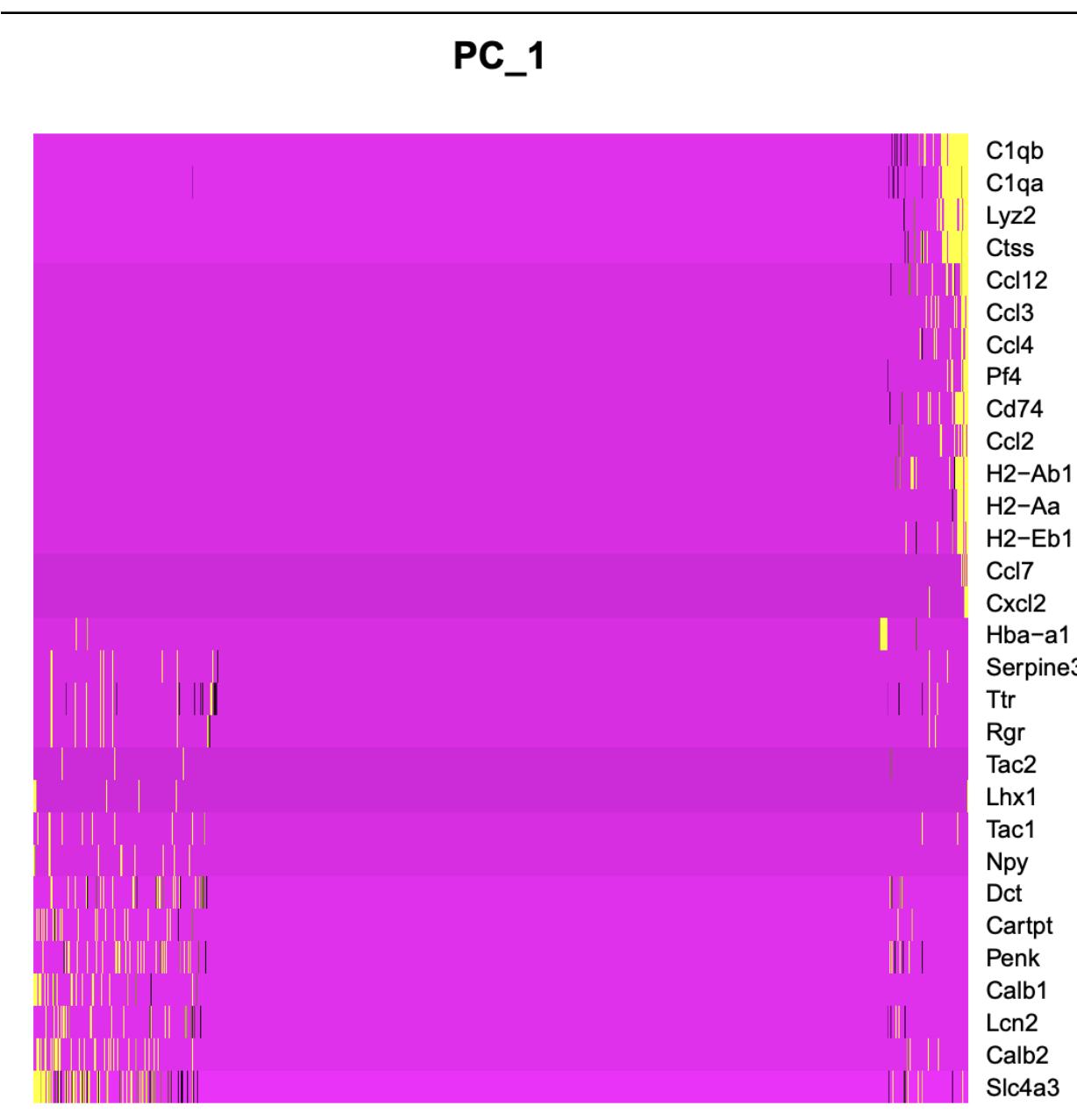
Highly Variable Gene (HVG) Selection

- Seurat default is 2000
- DNB Bioinformatics core default typically 3000
- Won't necessarily change this parameter
- Be aware number of HVGs impacts downstream results
- Always rerun after merging multiple samples

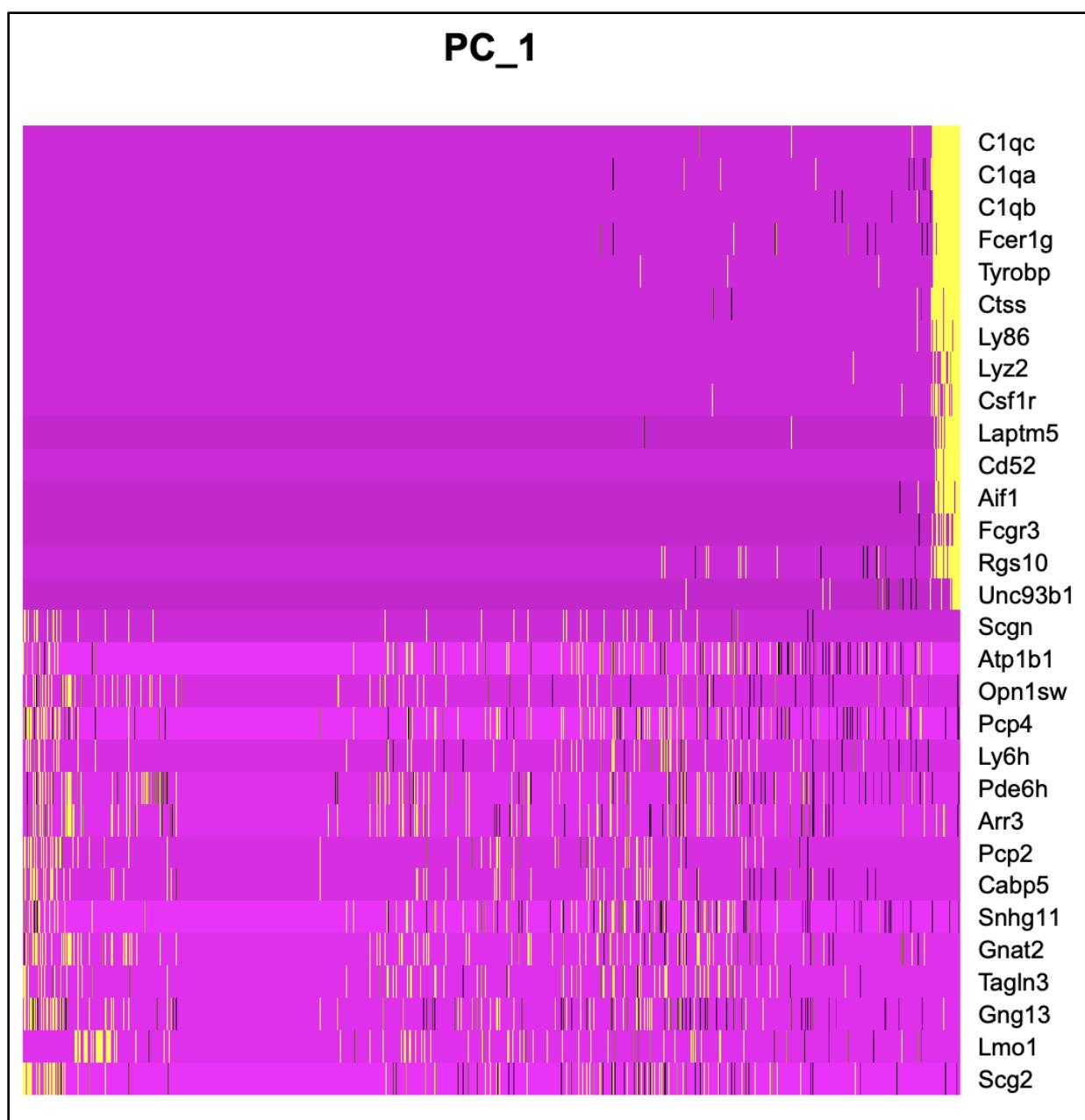


Highly Variable Gene (HVG) Selection

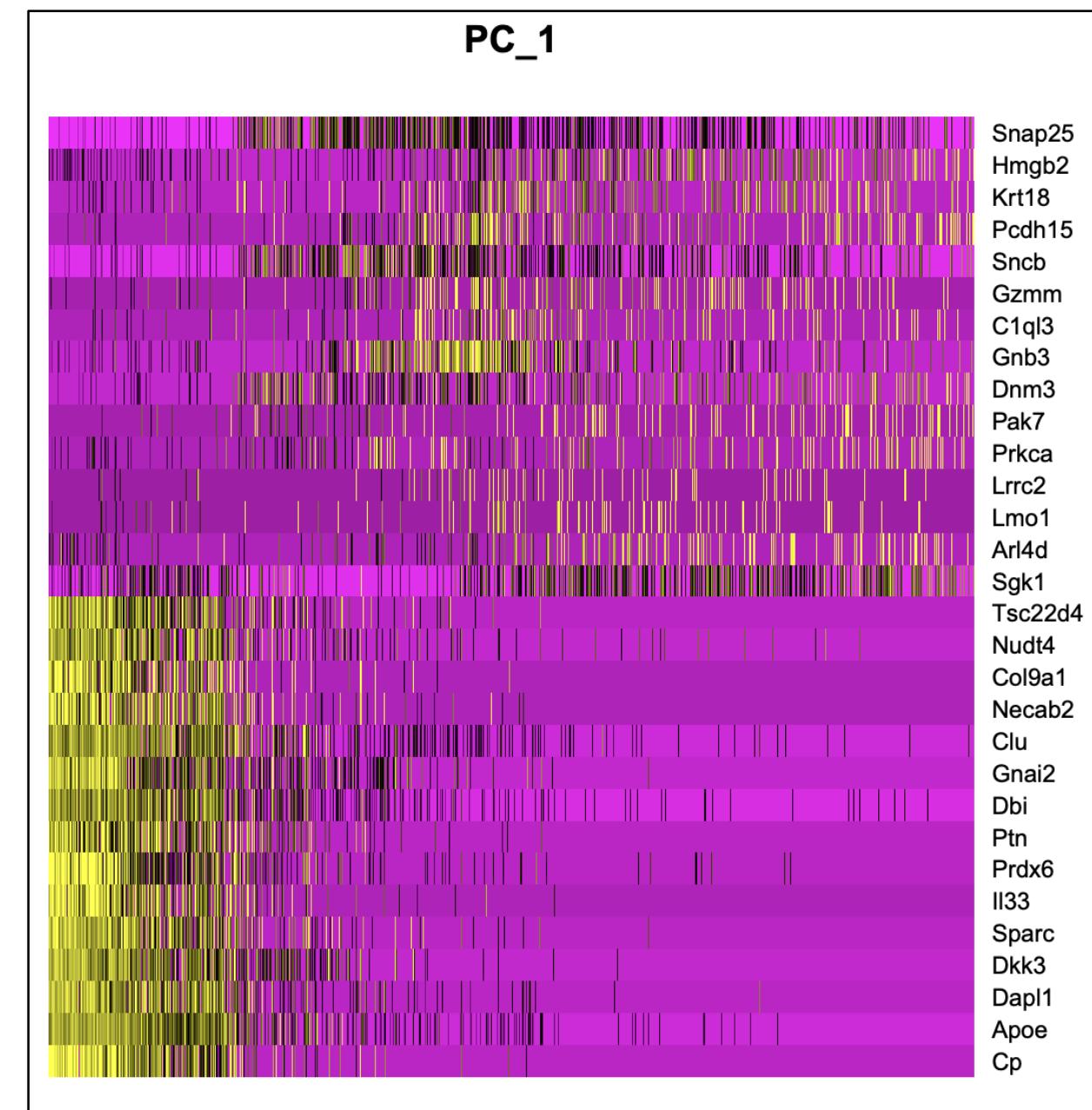
of HVGs = 30



of HVGs = 300

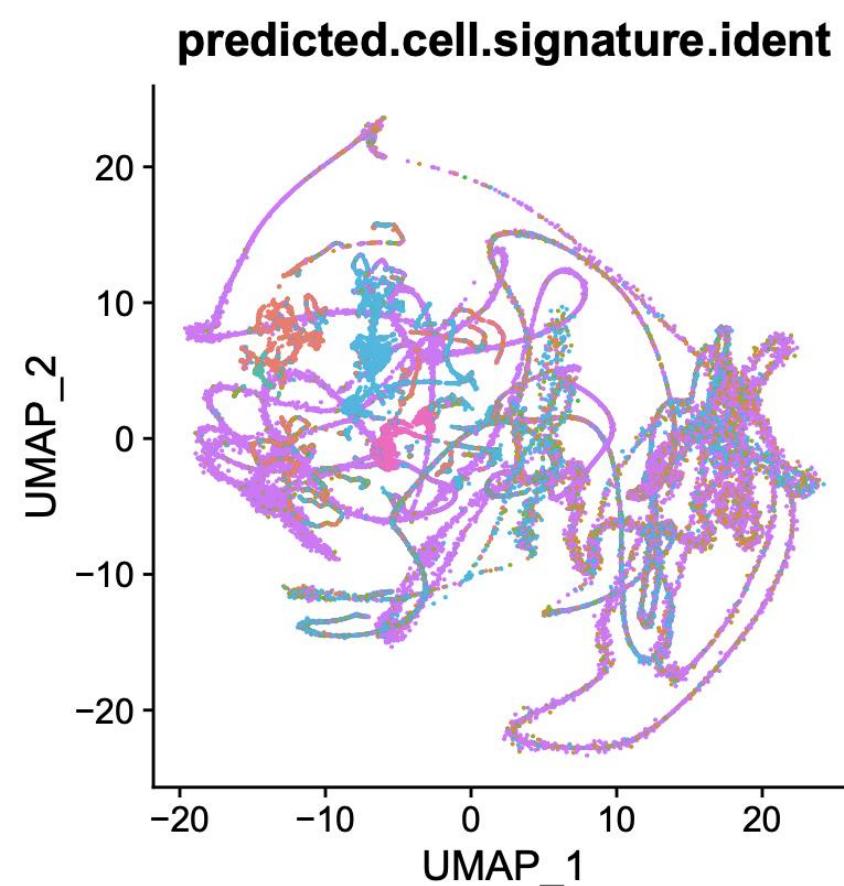


of HVGs = 3000

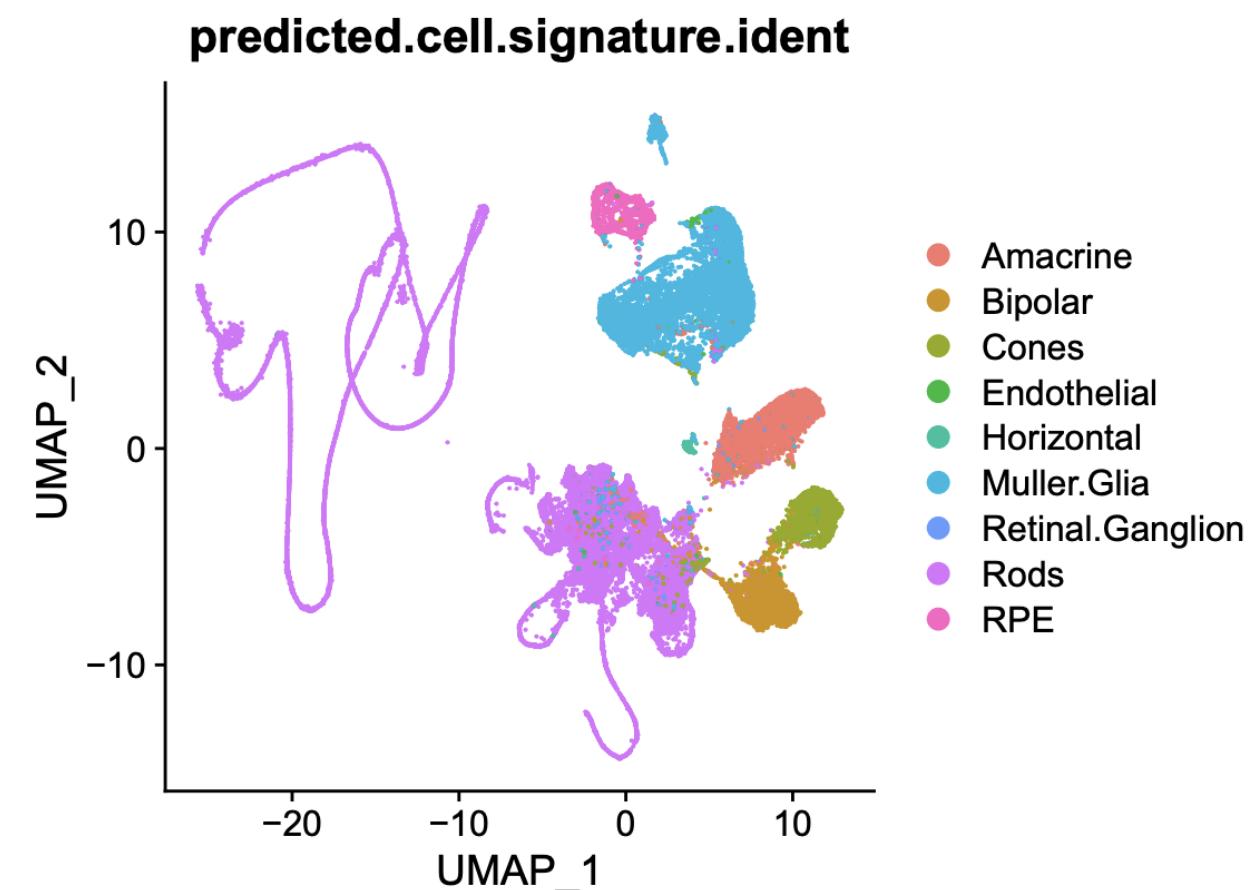


Highly Variable Gene (HVG) Selection

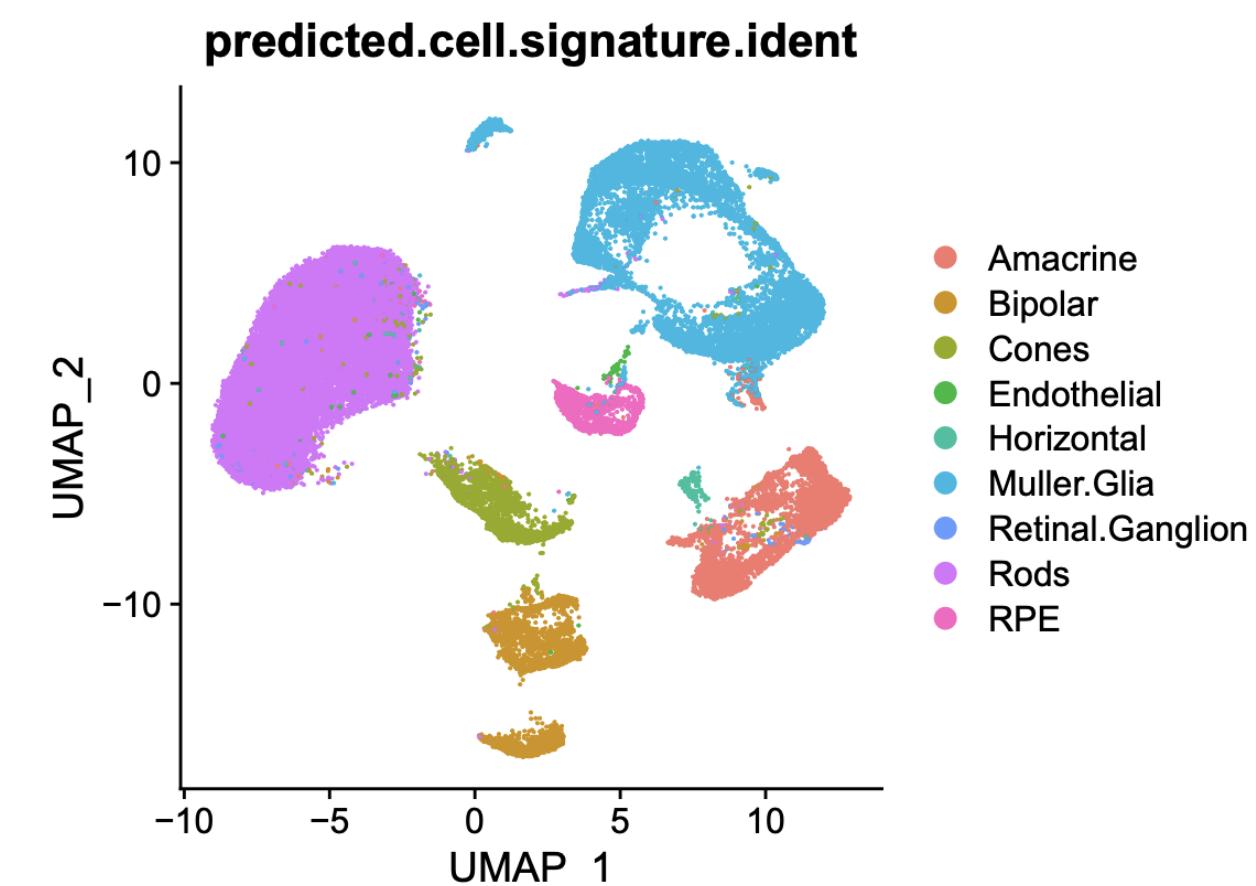
of HVGs = 30



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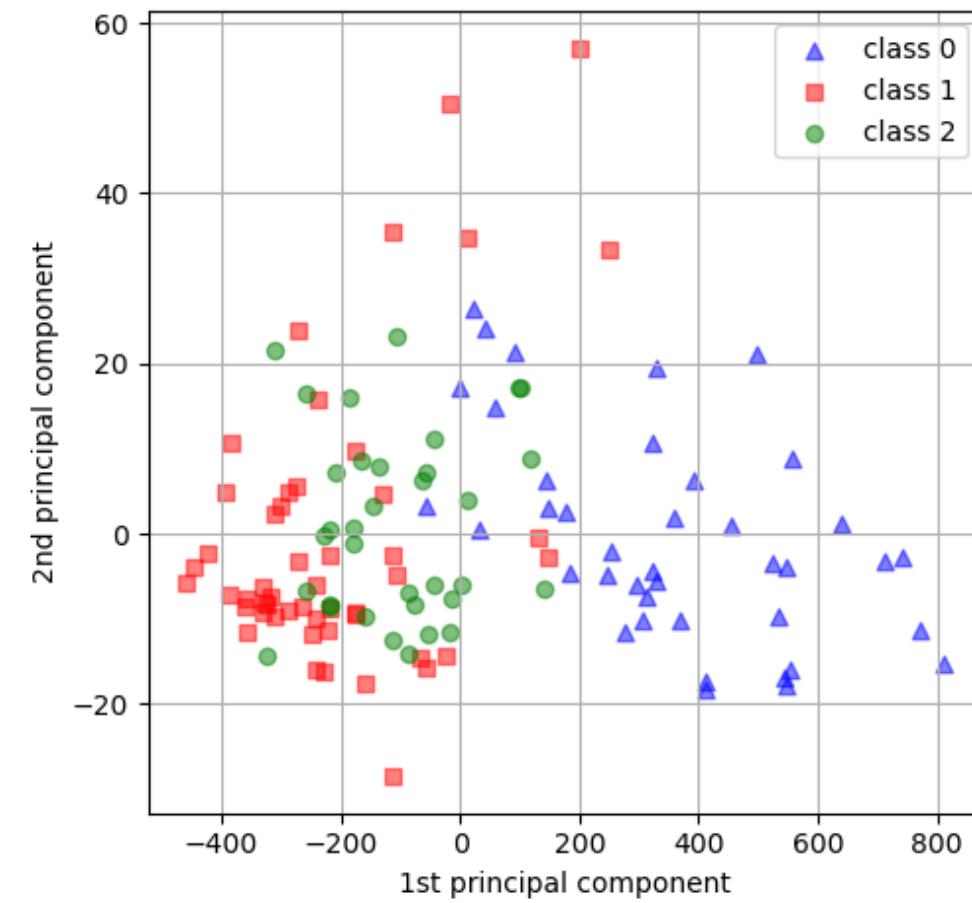
of HVGs = 3000



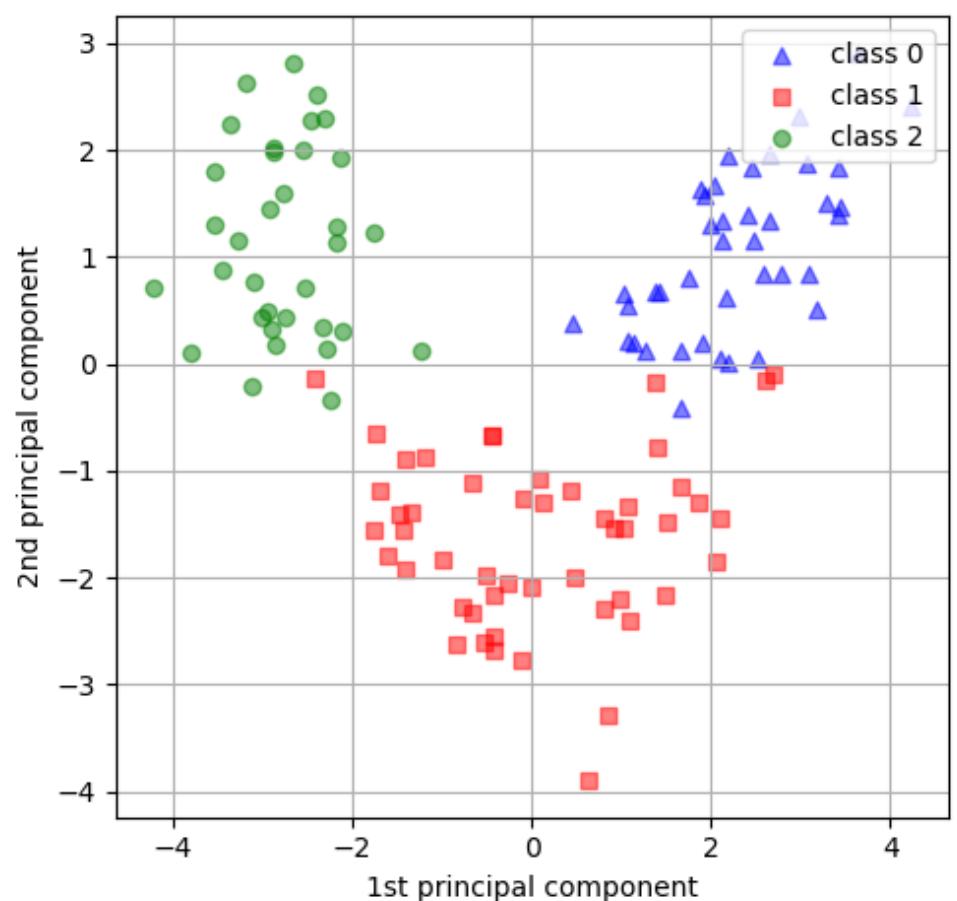
Scaling

- Scaling performed per gene
- Makes gene expression more comparable across cells, samples
- Reduces dominance of highly expressed genes
- Important for principal component analysis, downstream steps

PCA on unscaled data



PCA on scaled data



[Importance of Feature Scaling.](#)



Scaling

- Center and scale expression of each gene
 - Mean = 0
 - Standard deviation = 1
 - z-score transformation
- Scale before running PCA
- Always scaling rerun after merging samples
- Not rescaling can contribute to batch effects, technical artefacts



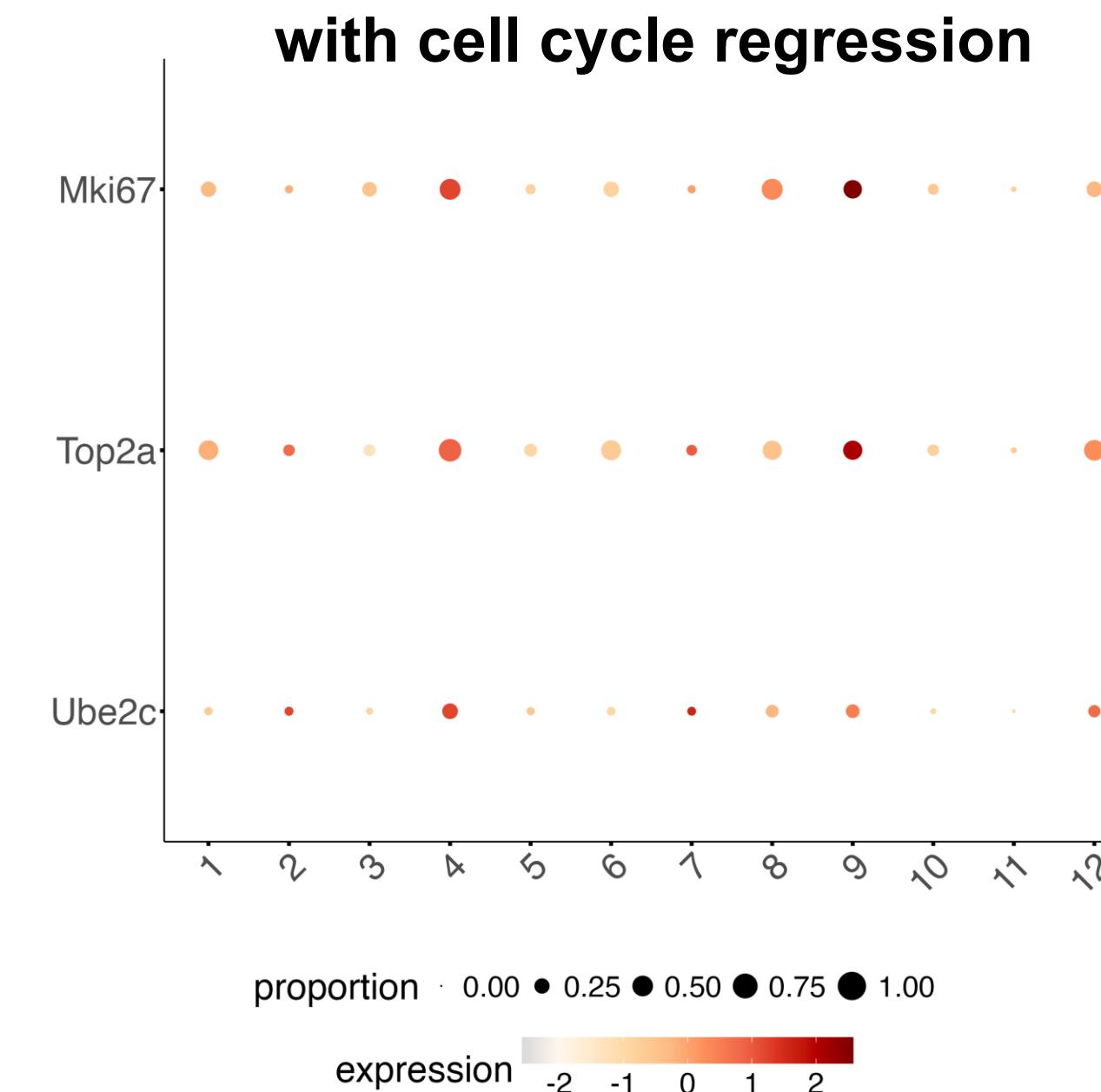
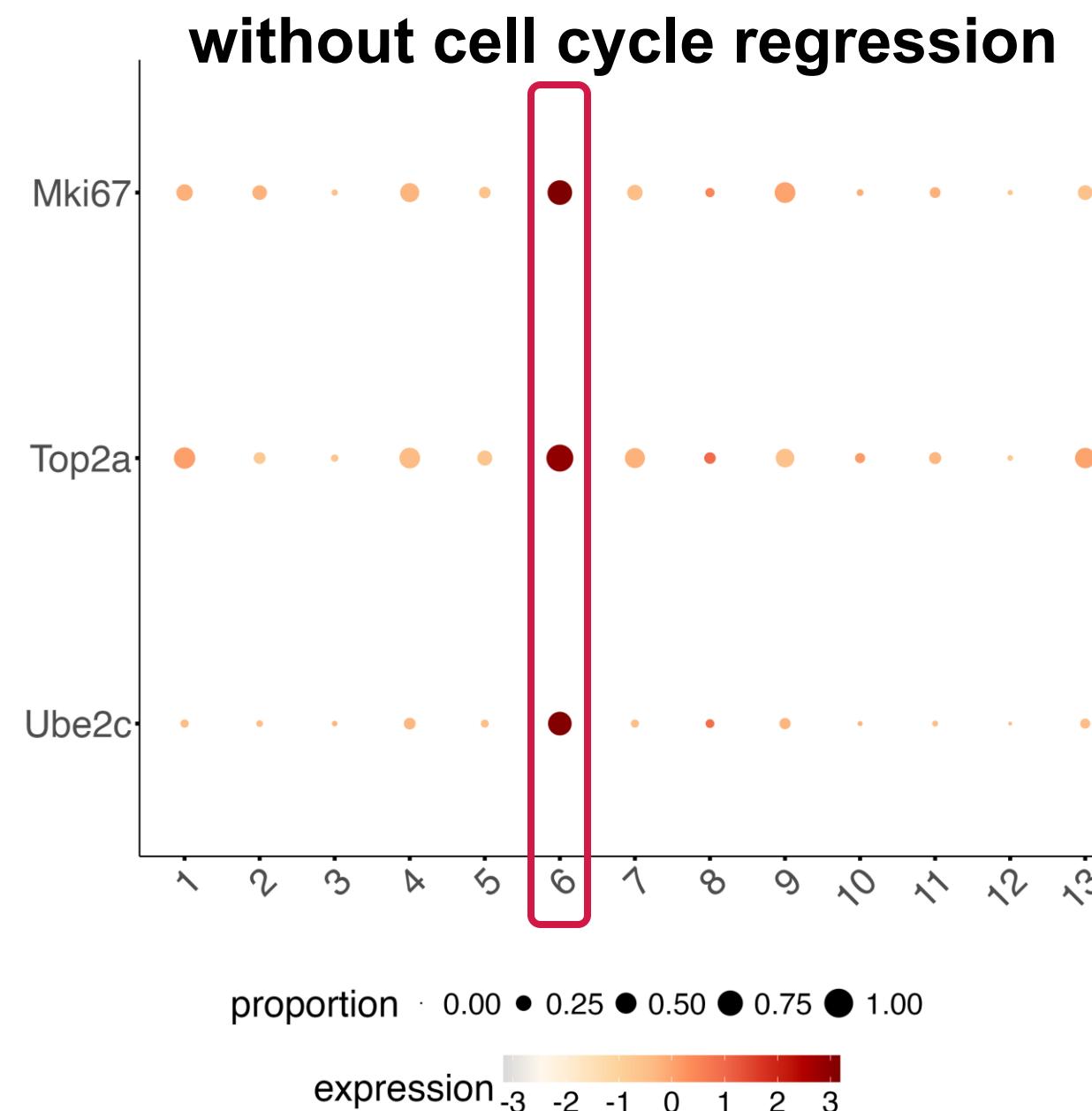
Scaling and Regression

- Can do regression during scaling step
- Regress out technical or biological variables
 - Cell cycle genes, mitochondrial genes, ribosomal genes, UMI count, batch
- Do analysis without regression first, add regression later if needed
- Seurat has three models for regression
 - Linear (default), Poisson (GLM), Negative Binomial (GLM)
- Regression can have unintended effects
- If regressing multiple variables, regress in same step

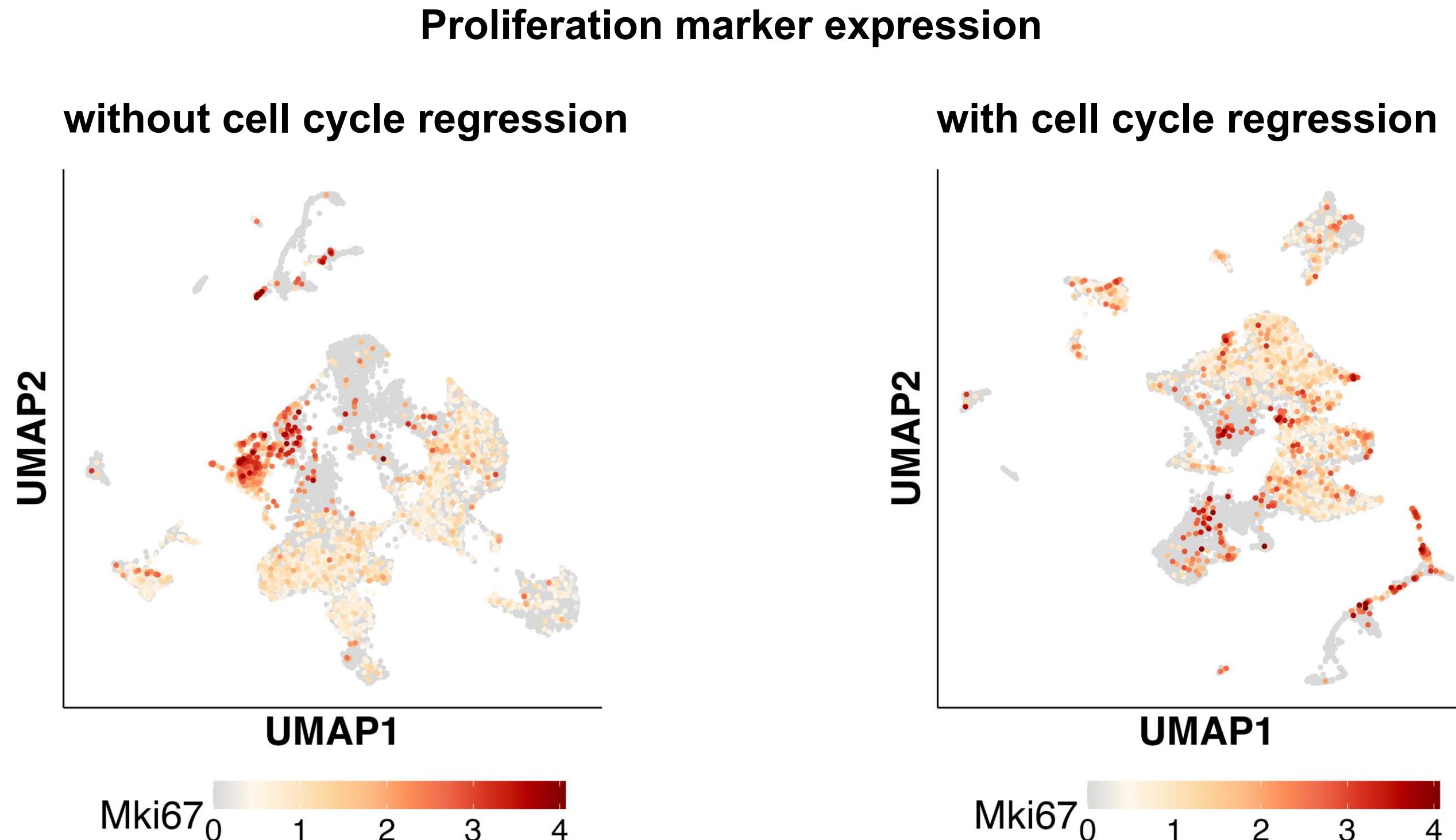


Scaling and Regression

Proliferation marker expression



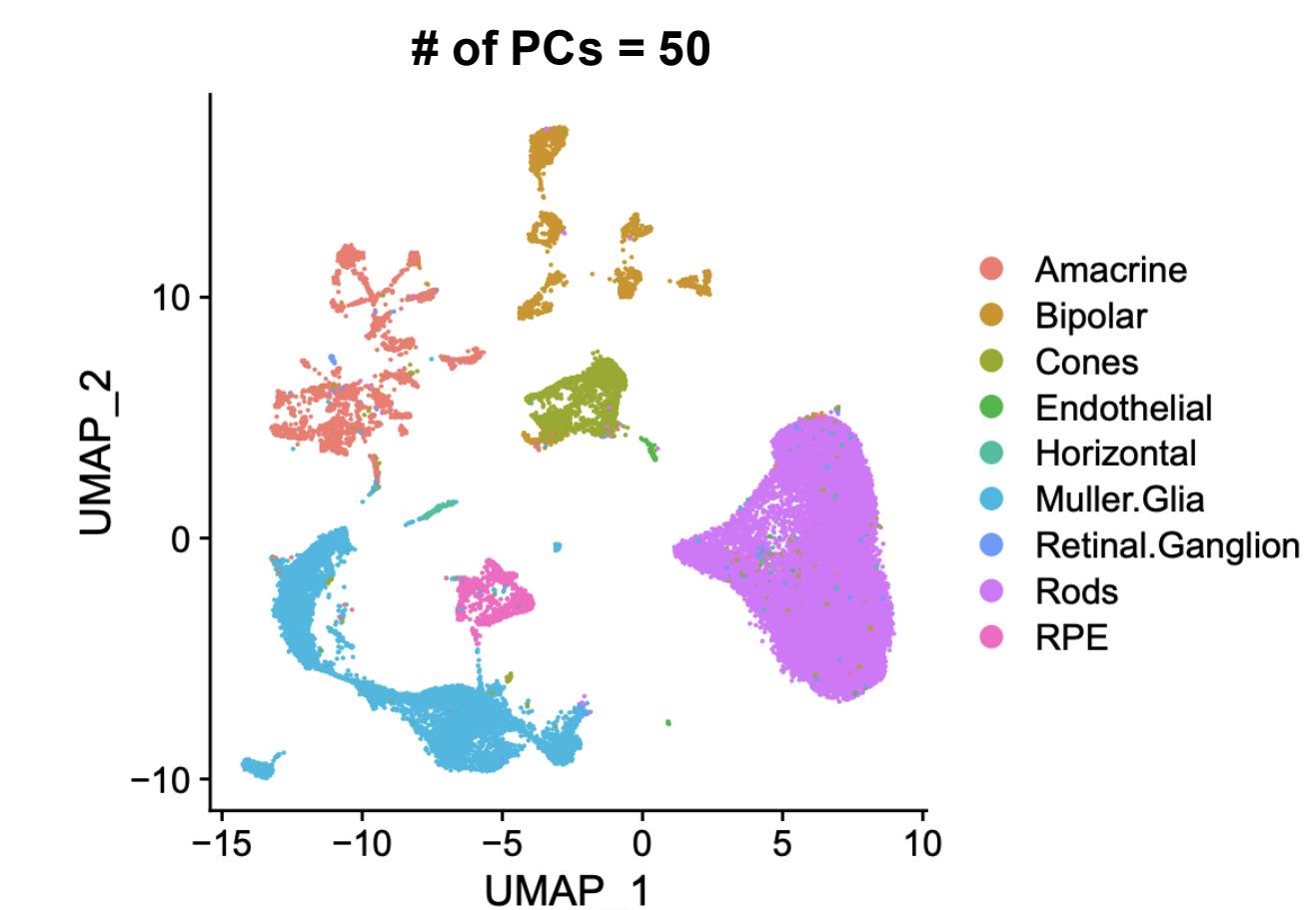
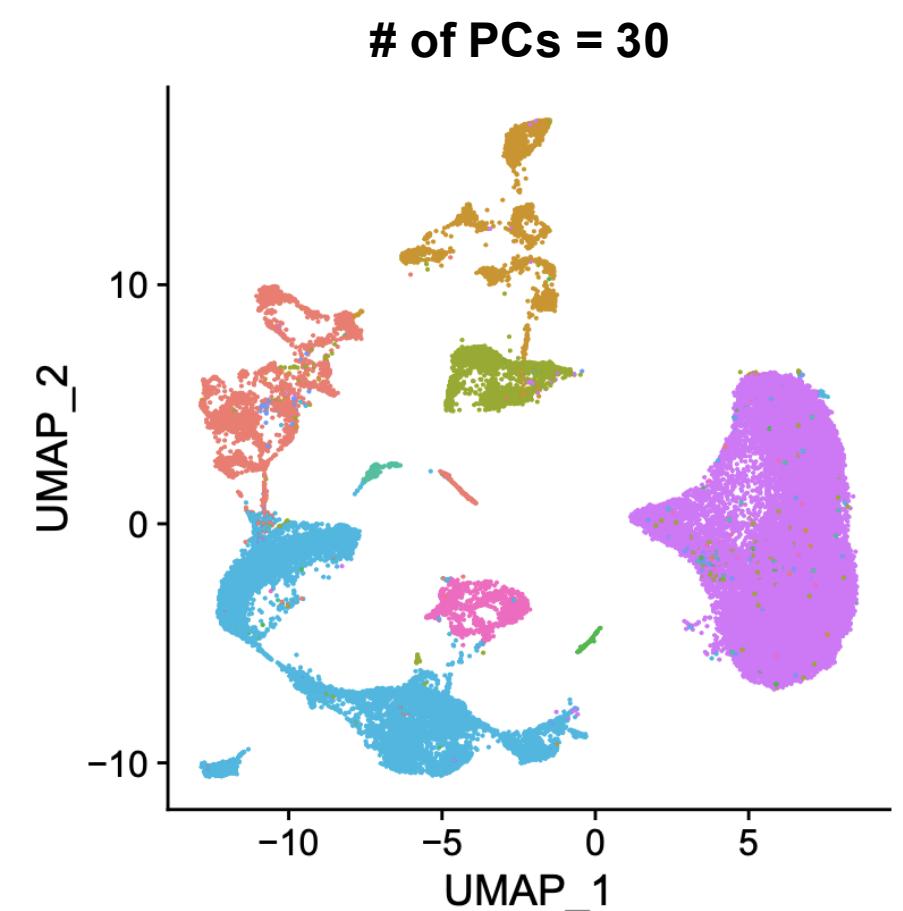
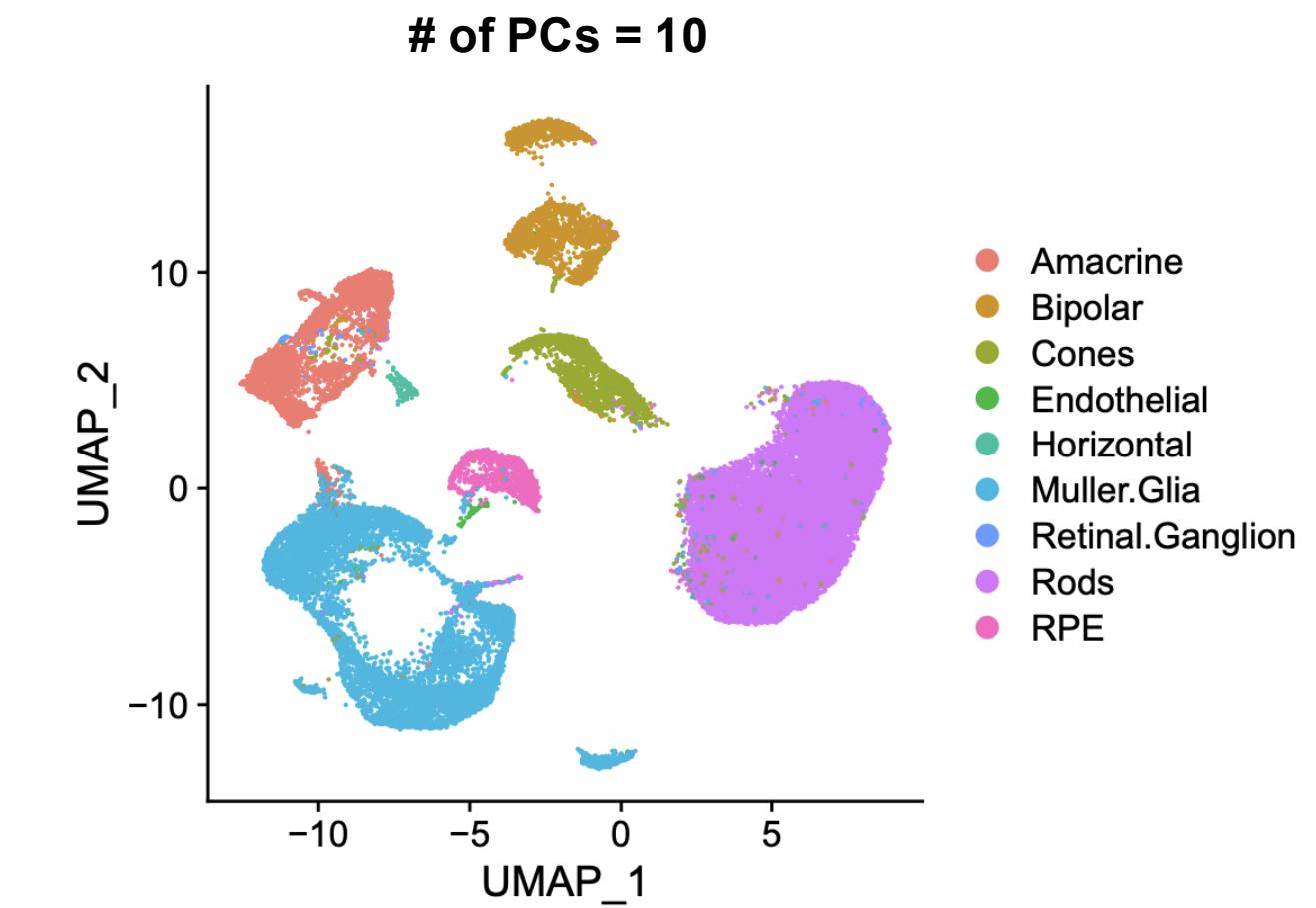
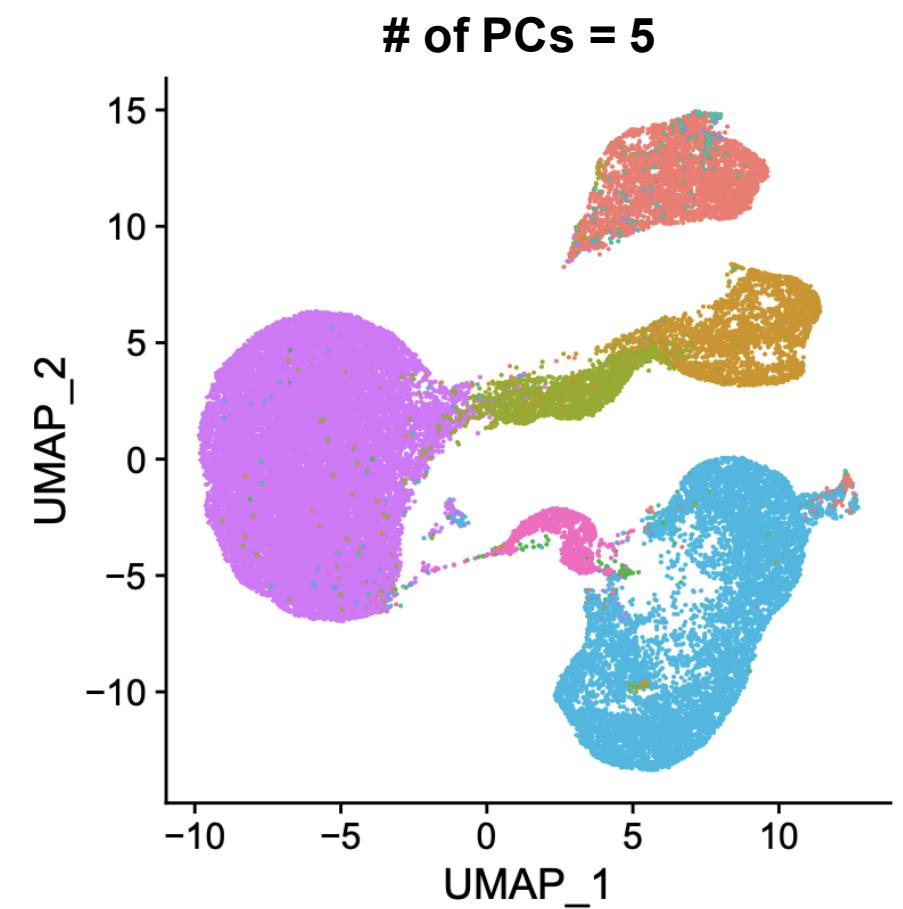
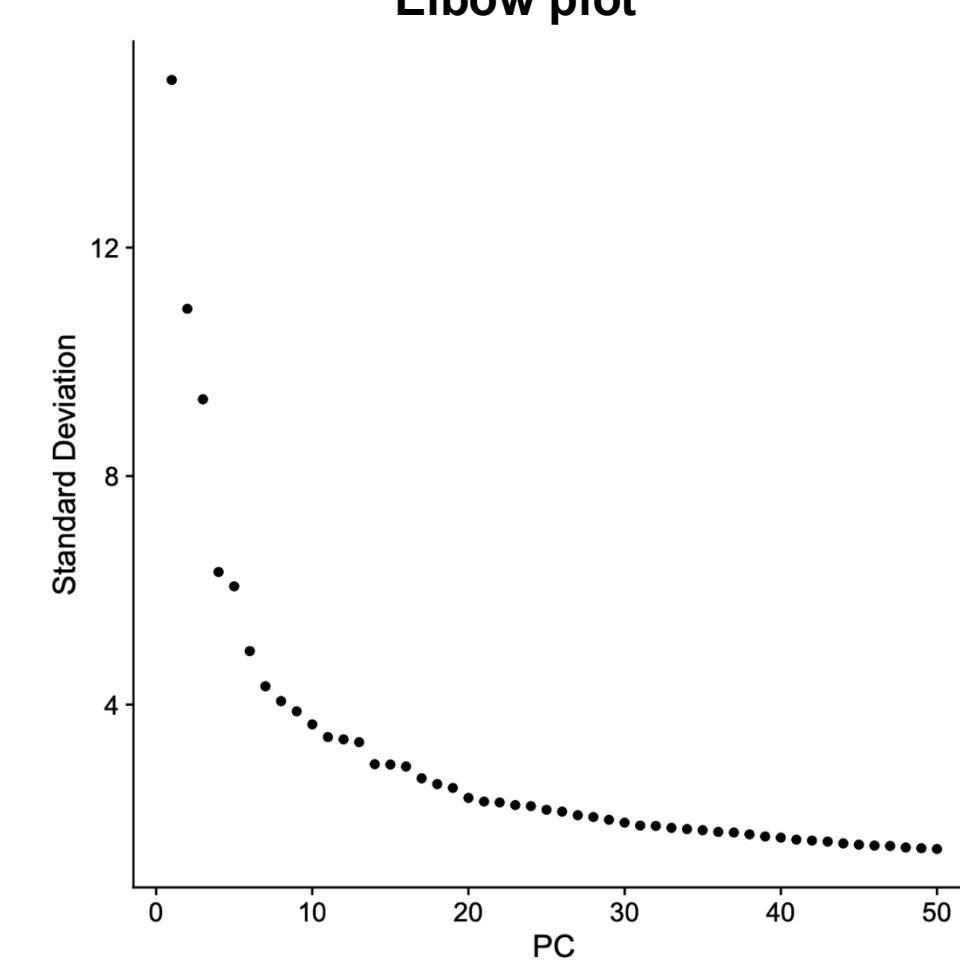
Scaling and Regression



PCA (linear dimensionality reduction)

- Principal component analysis (PCA) is common linear dimensionality reduction
 - Identify gene sets with correlated expression patterns
 - Capture heterogeneity across cells with fewer dimensions
- Choose an appropriate number of PCs
 - Too few, won't distinguish biology
 - Too many, include technical noise, have diminishing returns
- Sufficient range typically 10 to 50 PCs
- Use elbow plots for choosing reasonable number

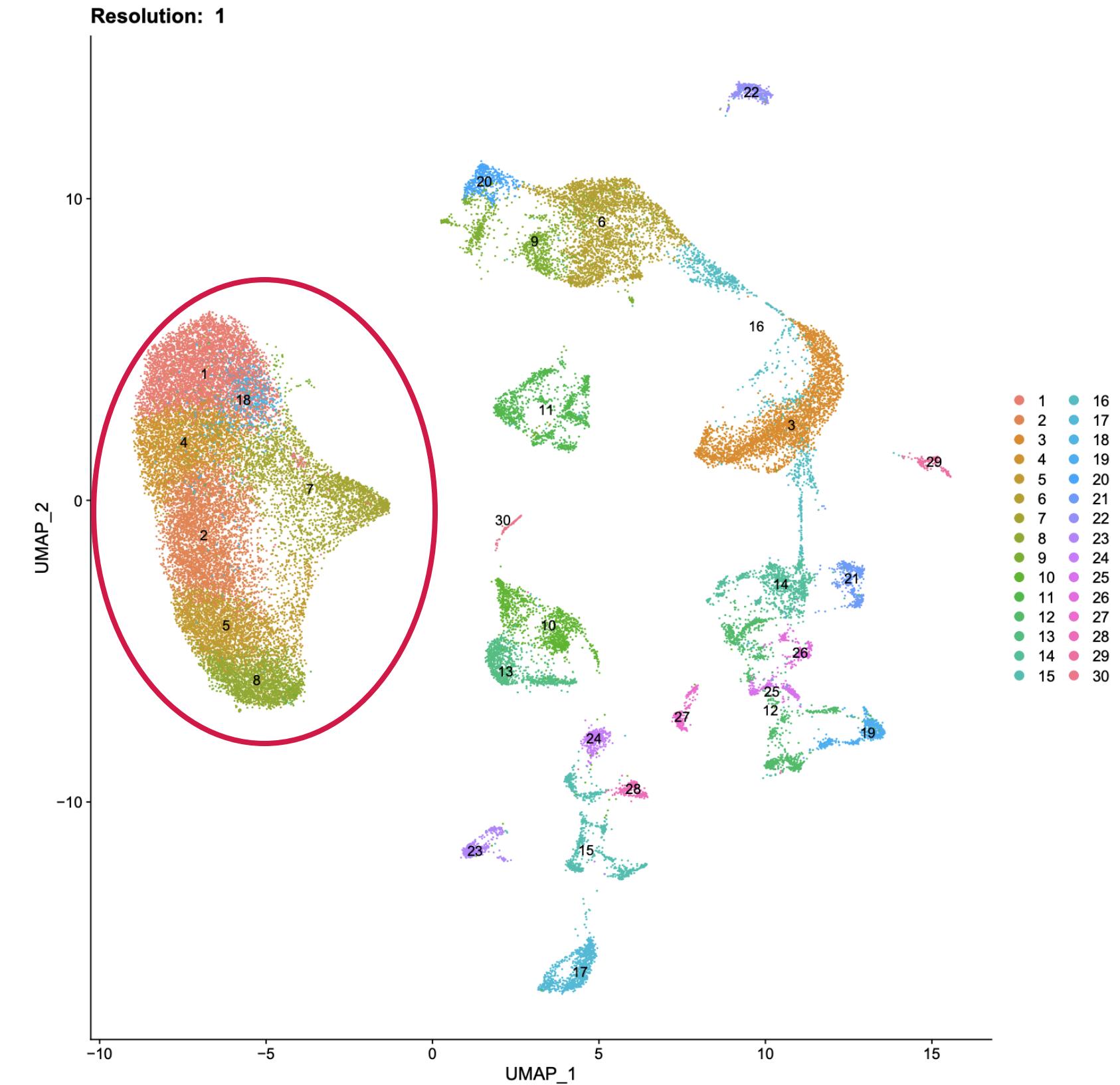
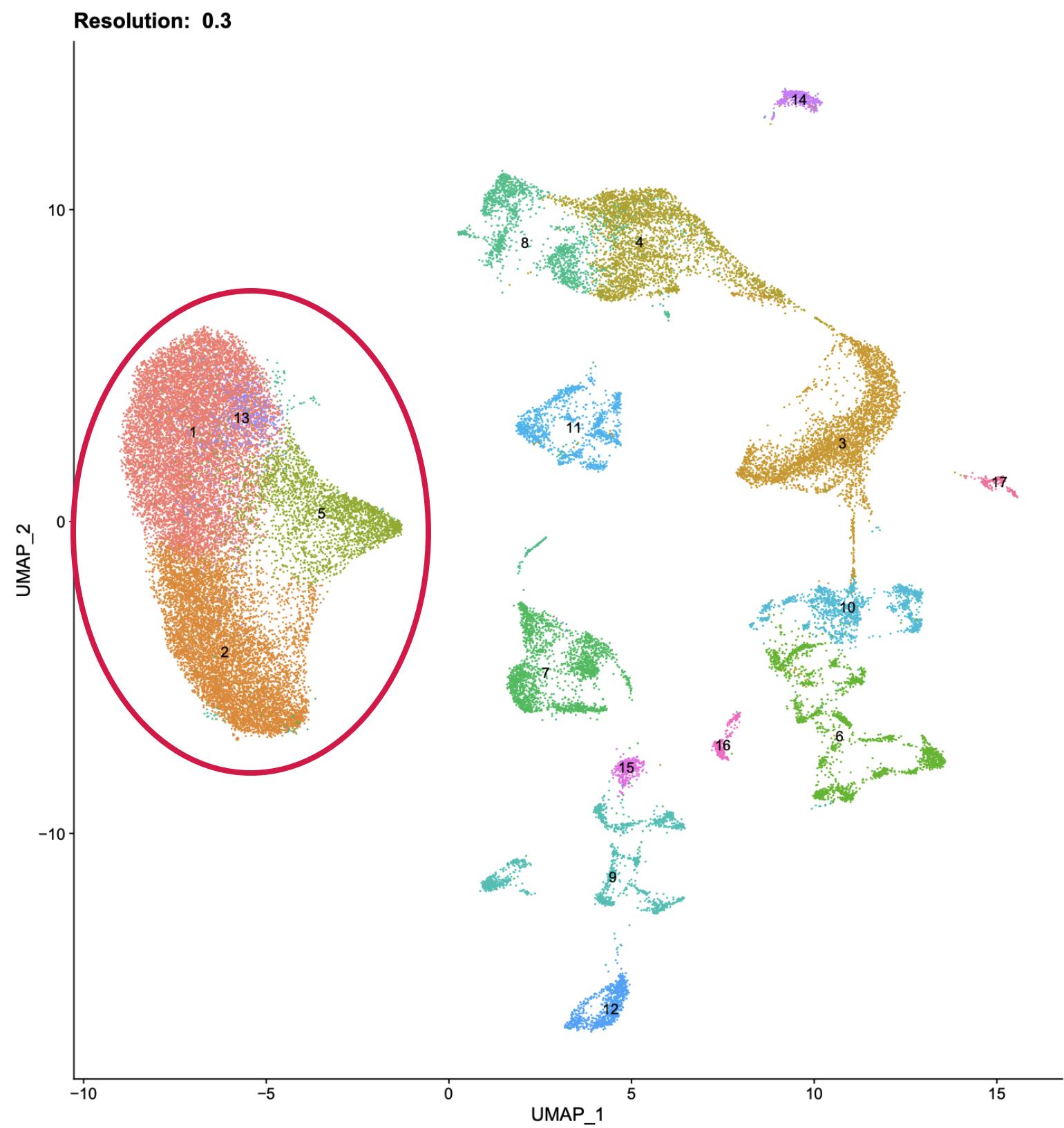




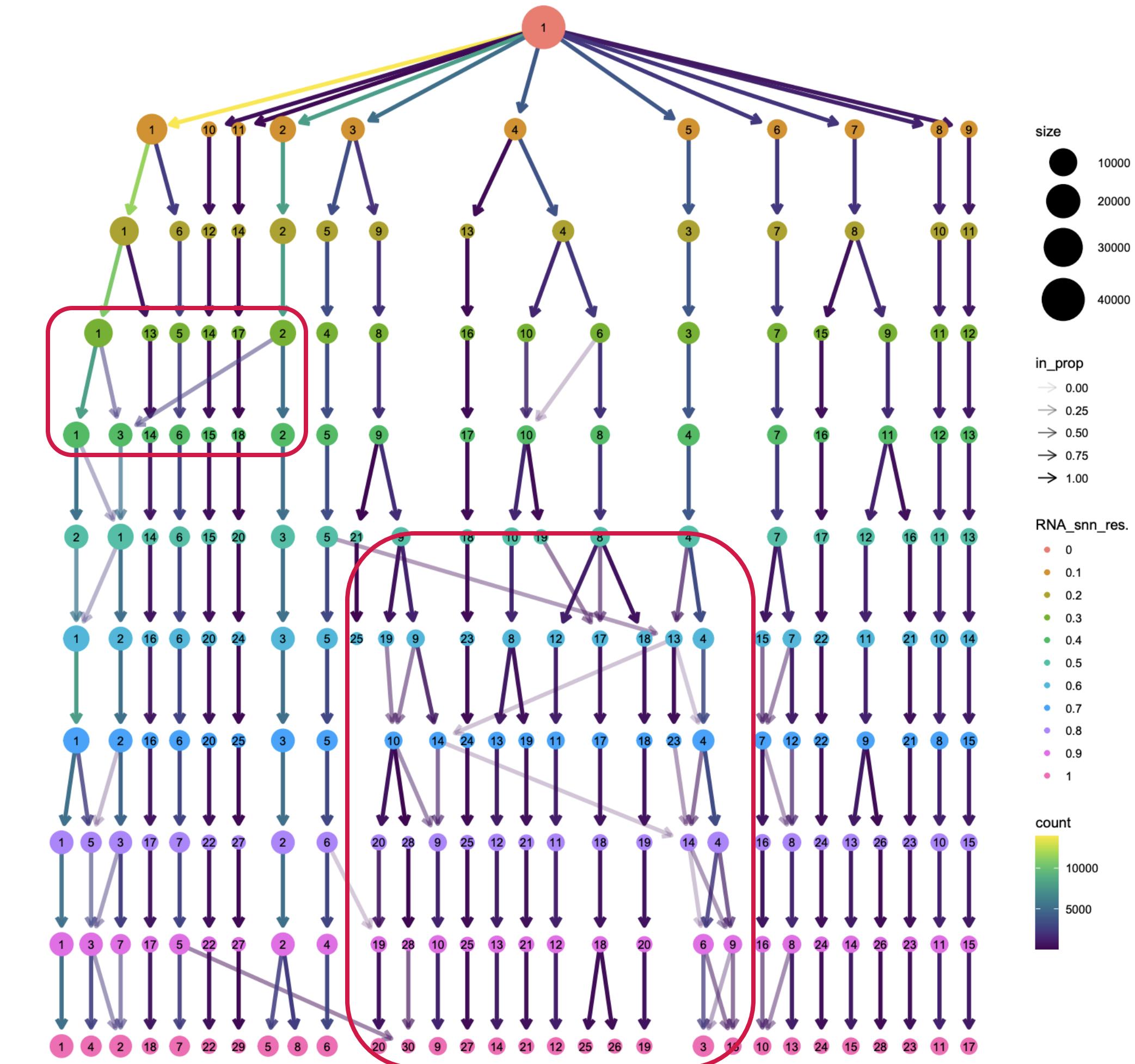
Clustering

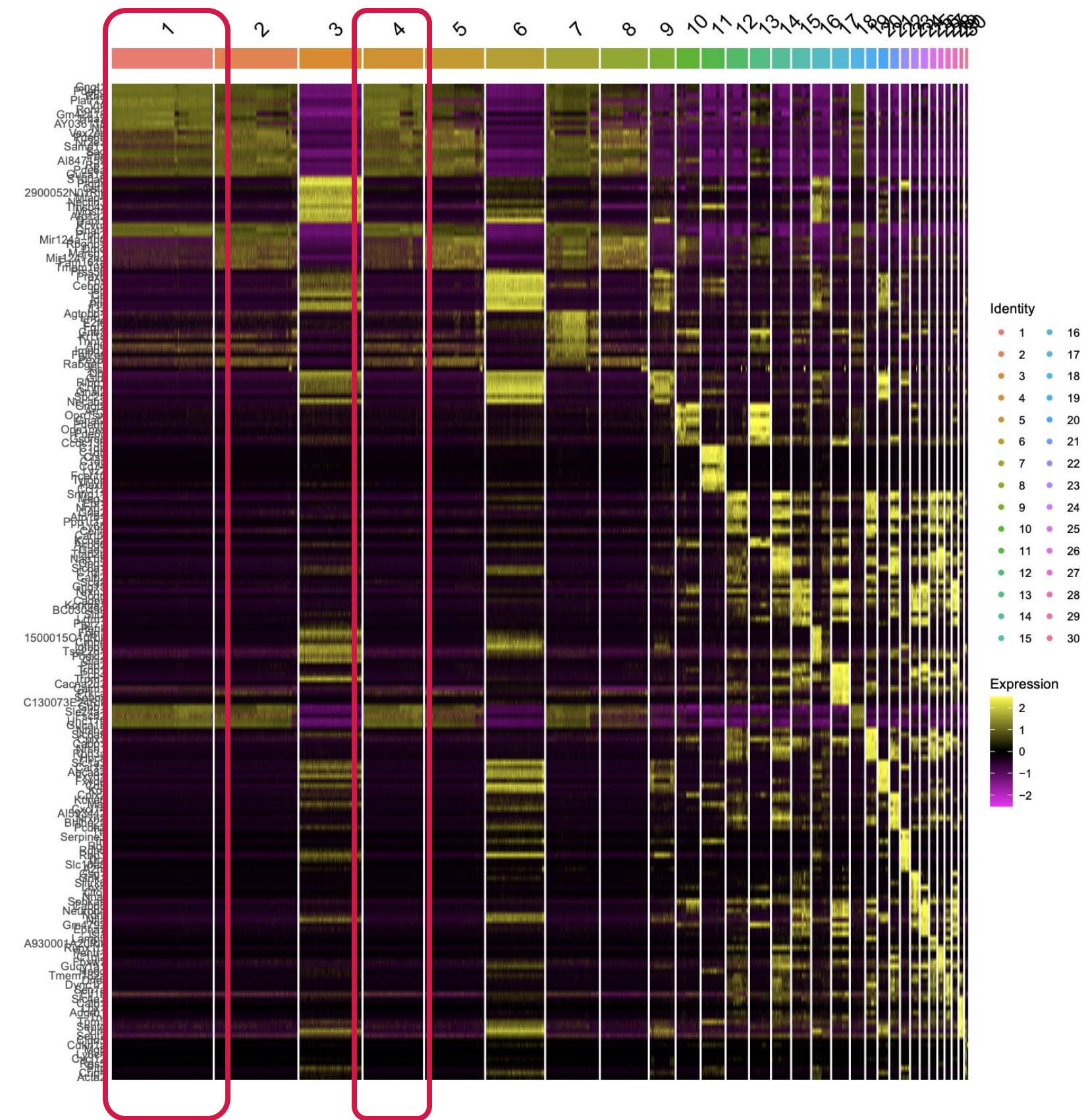
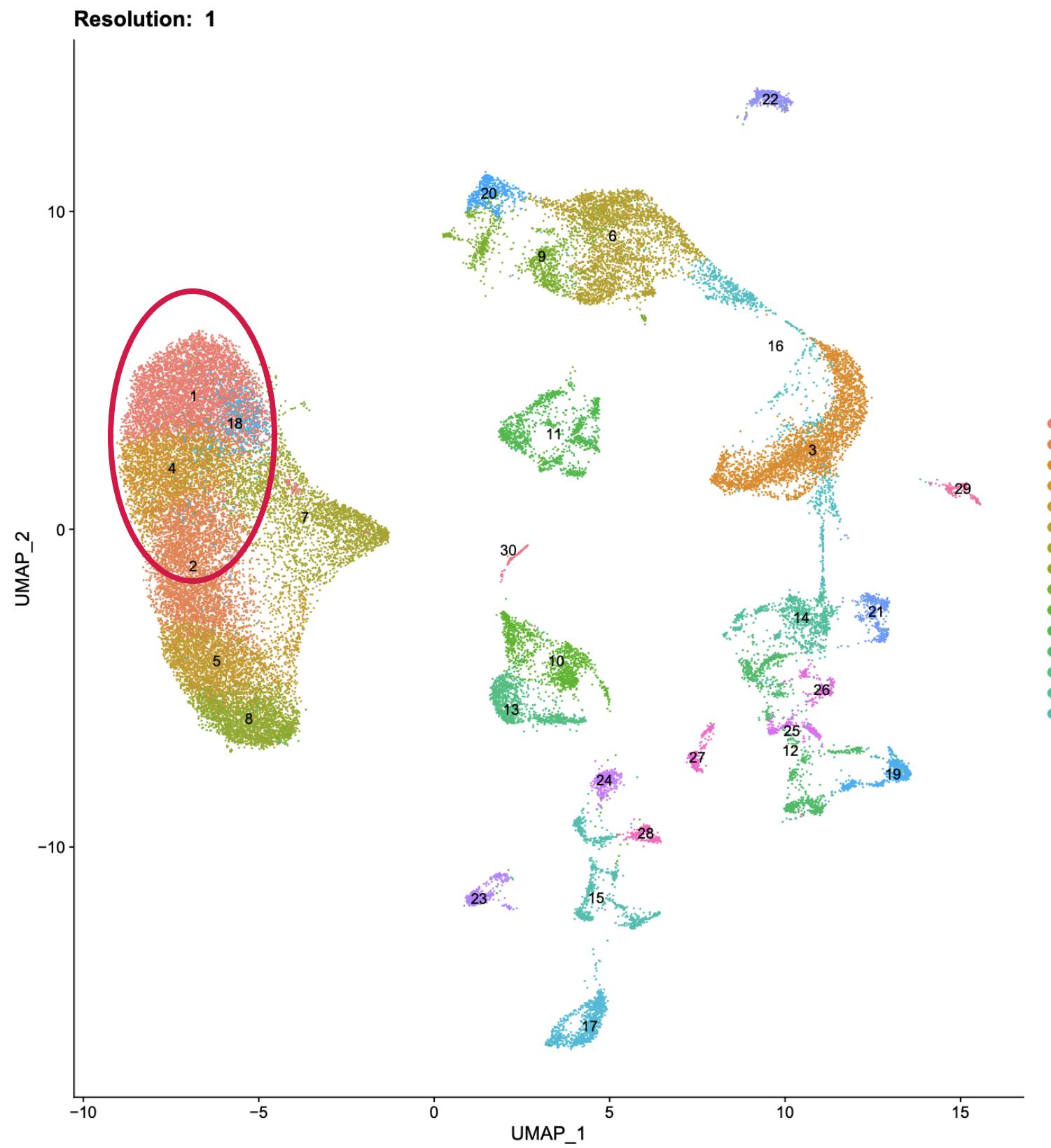
- Generating clusters is common next step after PCA
- Two steps in Seurat
 - FindNeighbors() – uses PCs, determines overlap in close cells
 - FindClusters() – uses FindNeighbors() similarity scores, groups cells optimally
- Important parameter for FindClusters() is resolution
- Resolution determines how broad or fine clustering is
 - Too low, group heterogenous cells together
 - Too high, split homogenous groups arbitrarily





- Each circle is a cluster
 - Size represents relative number of cells
- Each level corresponds to resolution
 - Resolutions 0 to 1, increments of 0.1
- Arrows represent how cells split, move across clusters from one resolution to the next
- Multiple incoming arrows to cluster, cells jumping, not splitting suggest overclustering





Non-linear dimensionality reduction and visualization

- Non-linear dimensionality reduction for visualization common
 - UMAP
- Display cells in two dimensions, preserve underlying data structure
 - E.g clusters ideally group in UMAP
- Uses PCs, improves speed, loses some structure
- Non-linear dimensionality reduction methods are imperfect
- 2D cannot capture full picture of true structure

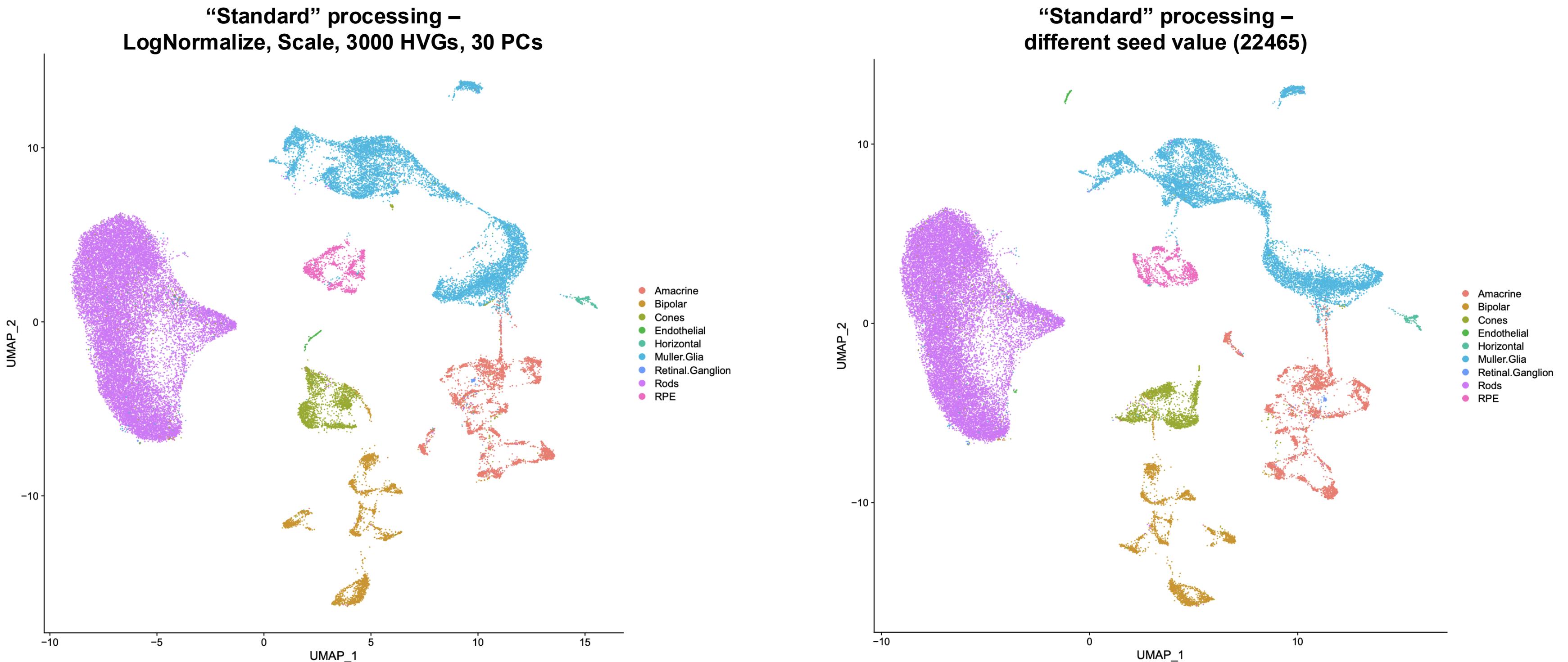


Non-linear dimensionality reduction and visualization

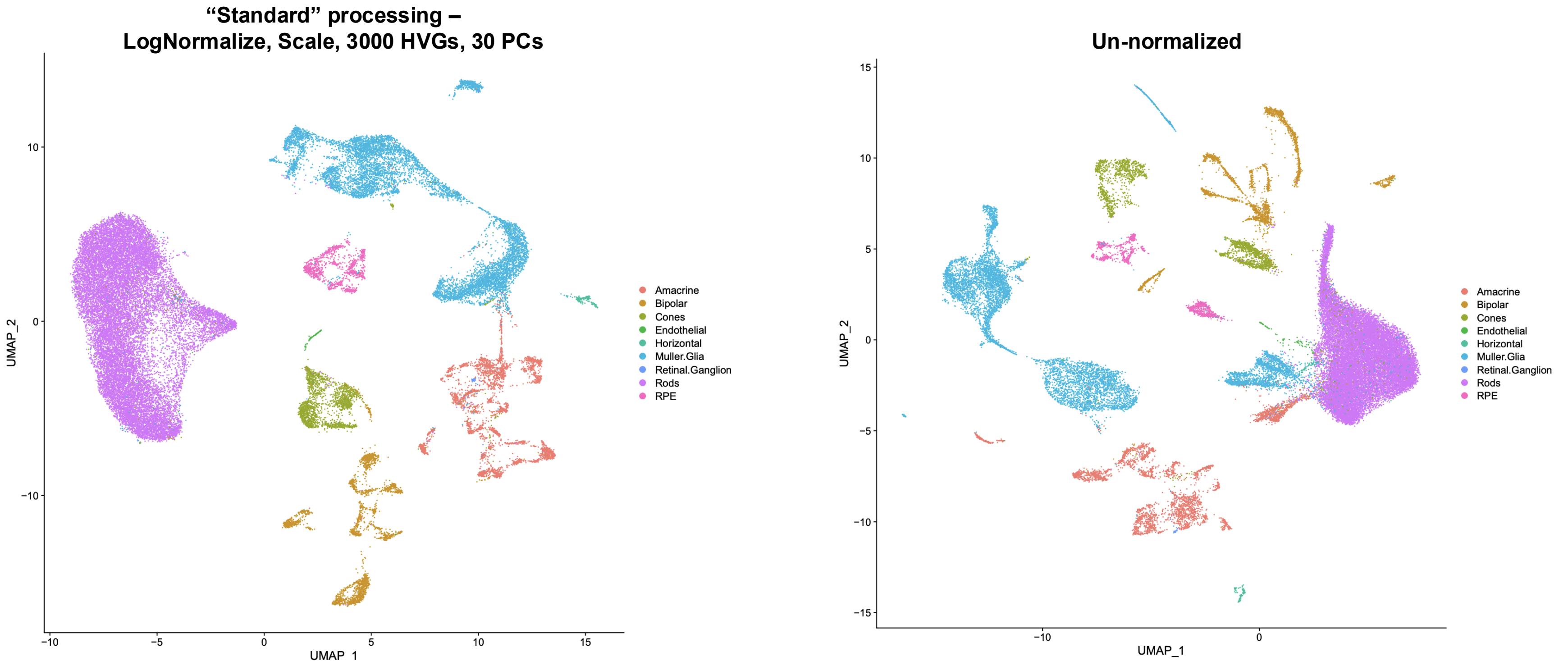
- Visualizations can help you refine data, e.g. patterns may suggest:
 - Ambient RNA contamination, doublets
 - Cells grouping on cell cycle
 - Cells grouping on filtering metrics
- Do not rely on UMAP for biological interpretation
- Shape can easily change based on numerous factors
 - Seed, normalization, scaling, variable features, principal components
- Structure may match biology, but verify through additional methods
- Don't force structure to fit biology



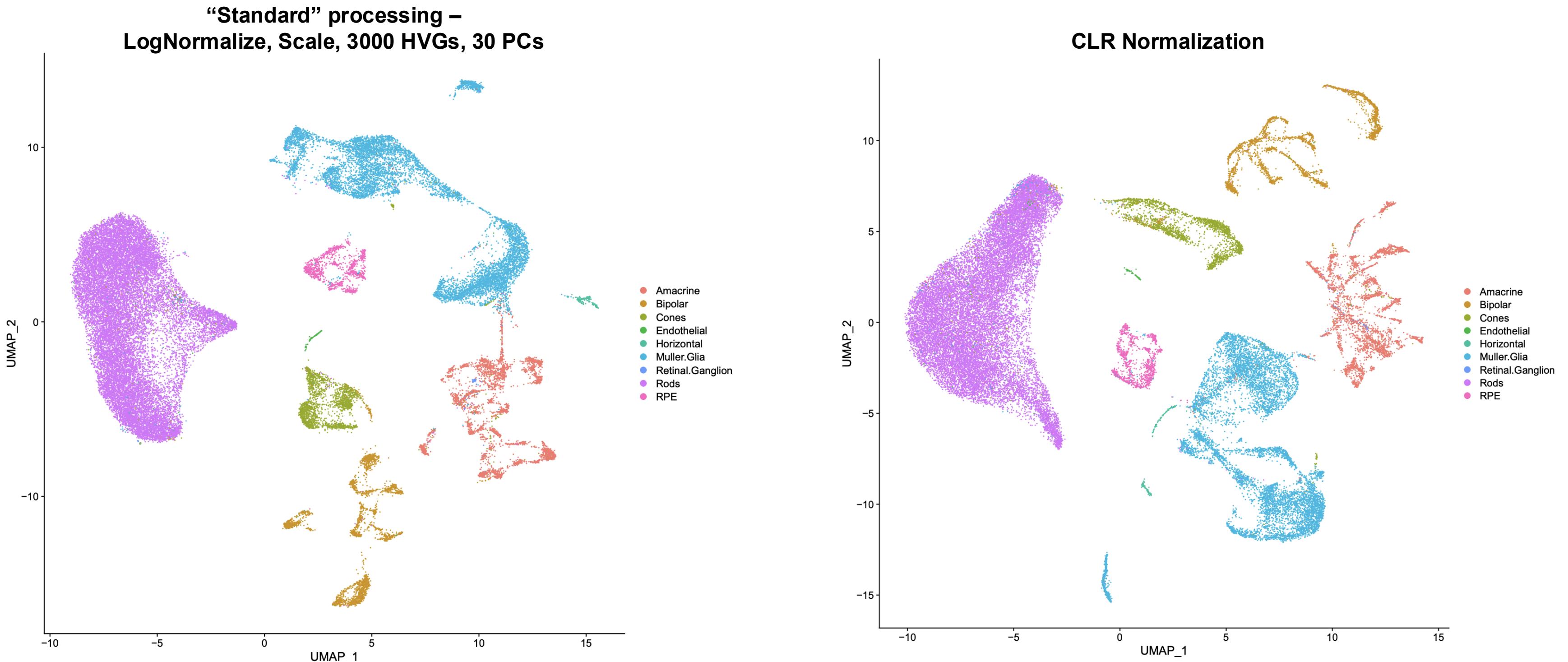
Non-linear dimensionality reduction and visualization



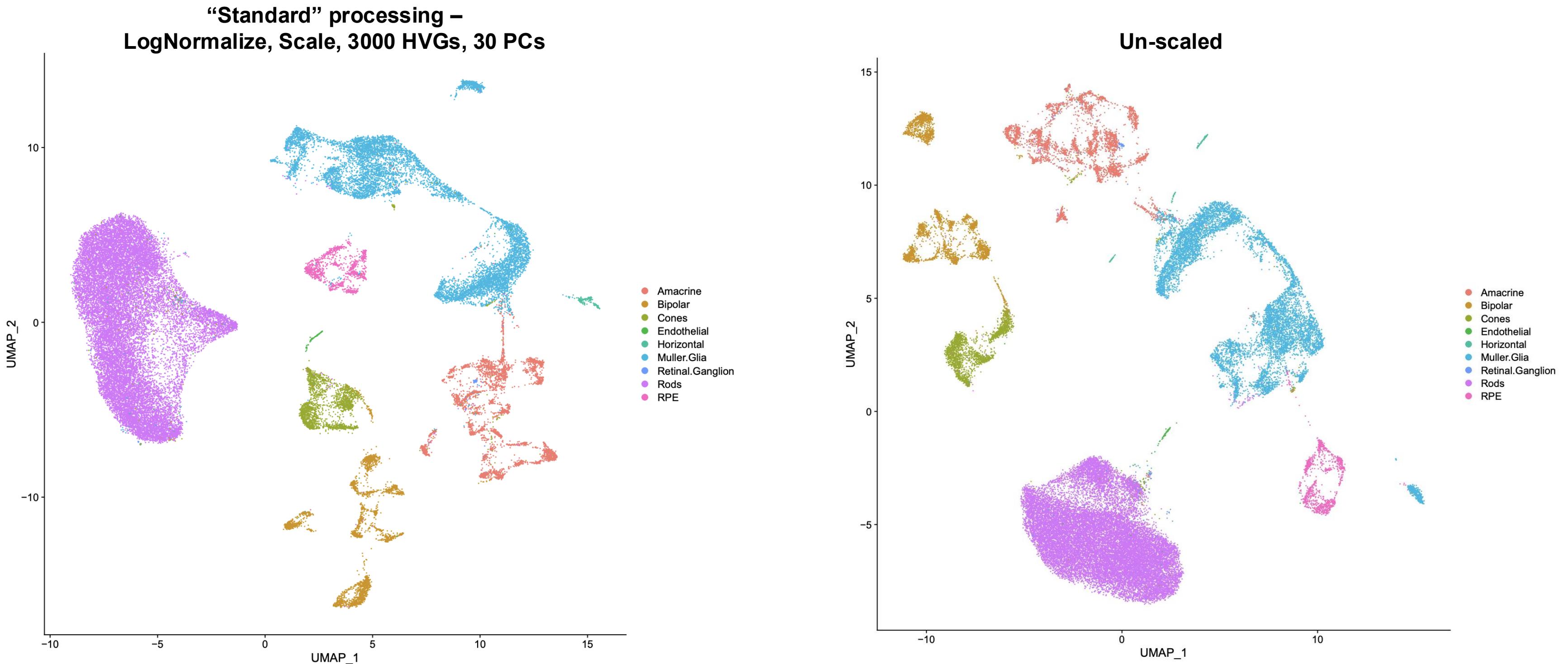
Non-linear dimensionality reduction and visualization



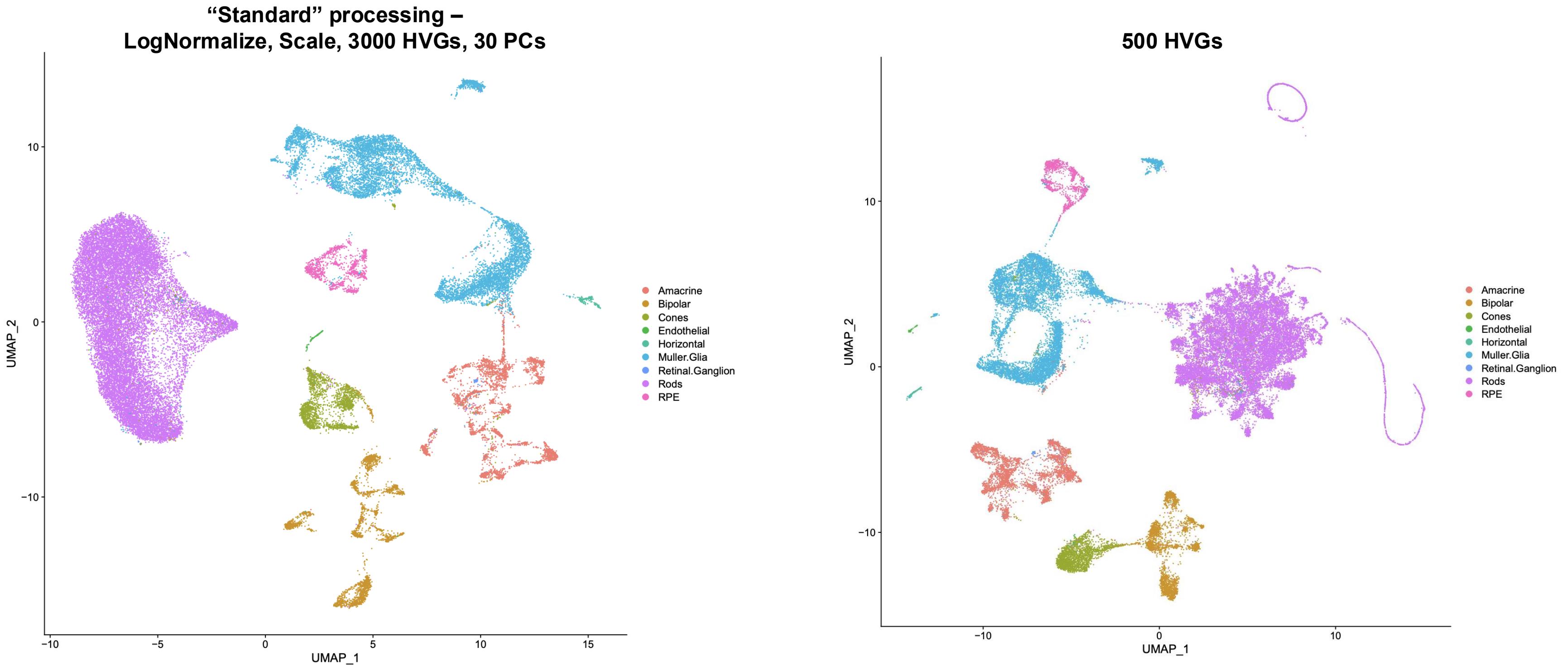
Non-linear dimensionality reduction and visualization



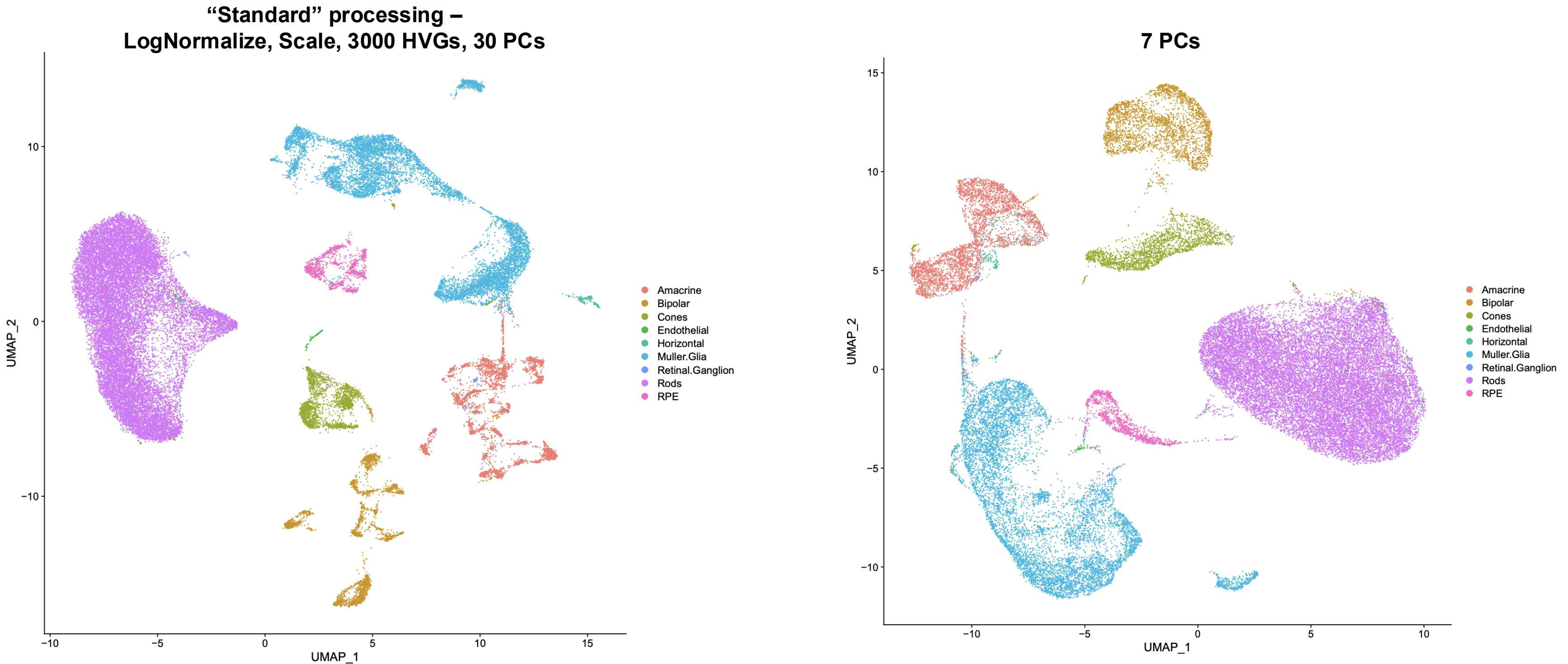
Non-linear dimensionality reduction and visualization



Non-linear dimensionality reduction and visualization

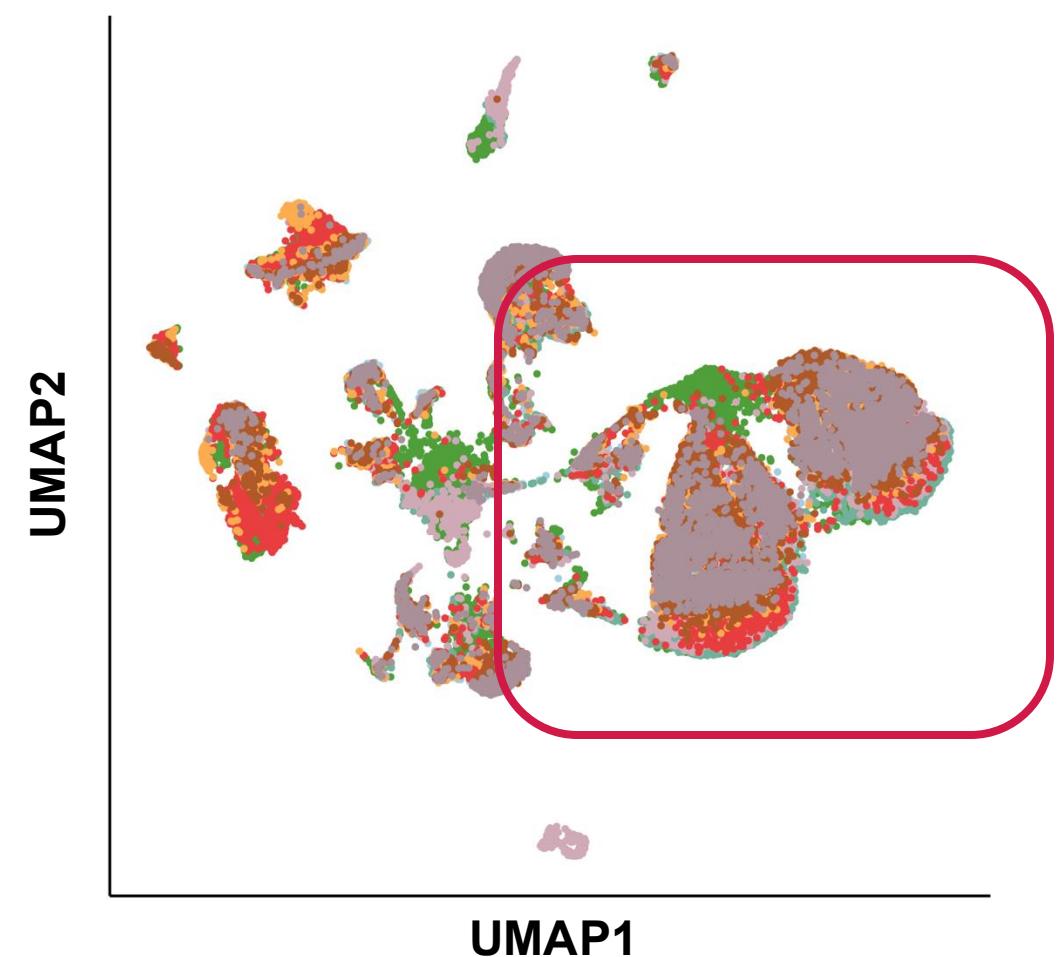
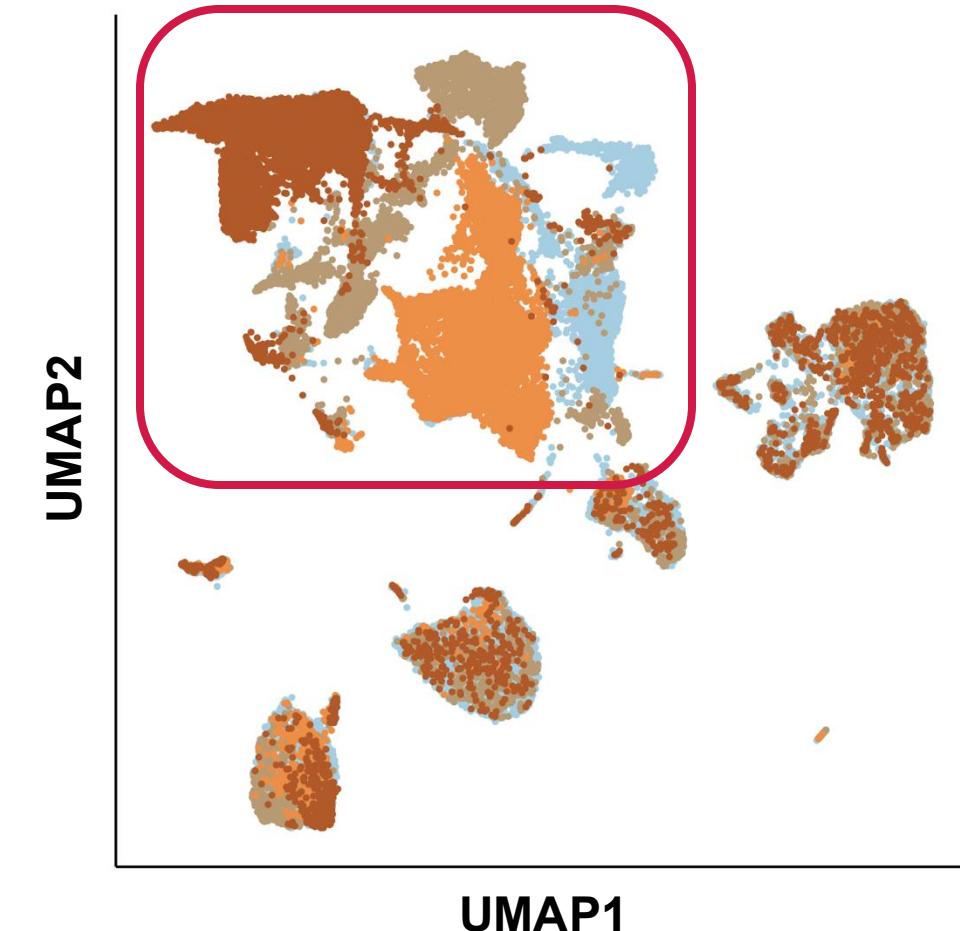


Non-linear dimensionality reduction and visualization



Integration

- Often group multiple samples for clustering, visualization, downstream analysis
- Simple merges show sample-based batch effects
 - Distinct cluster per sample
 - Rainbow effect within clusters

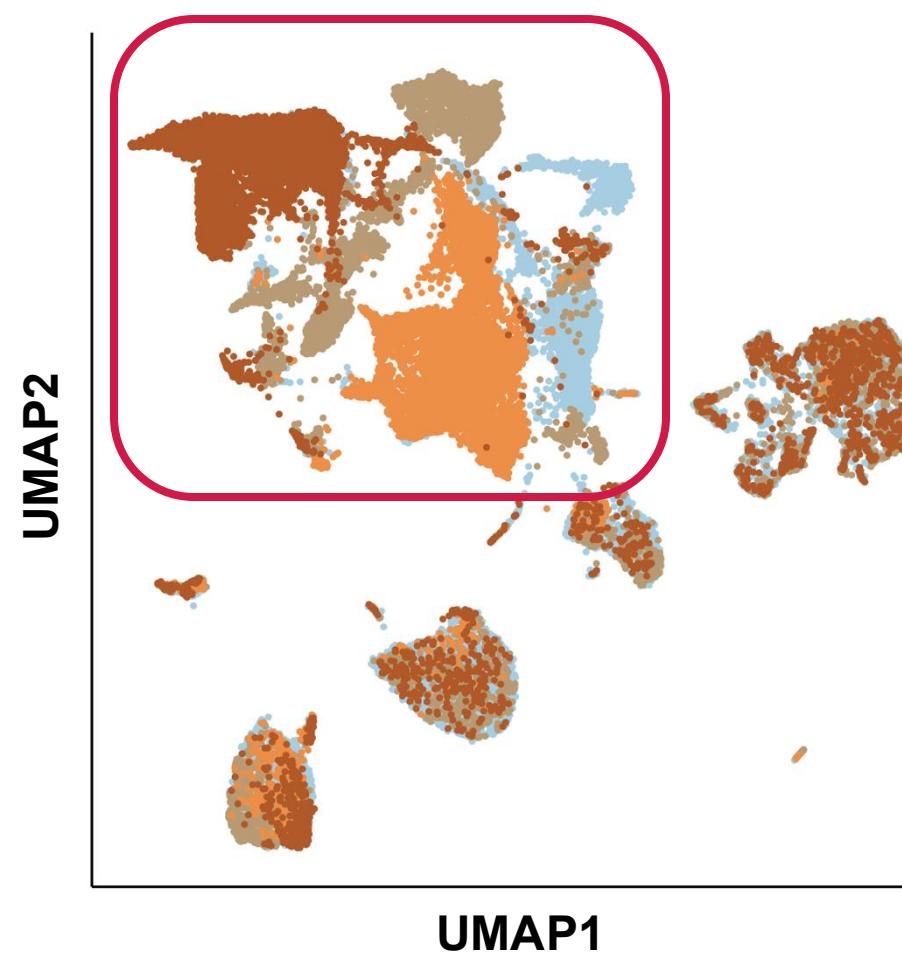


Integration

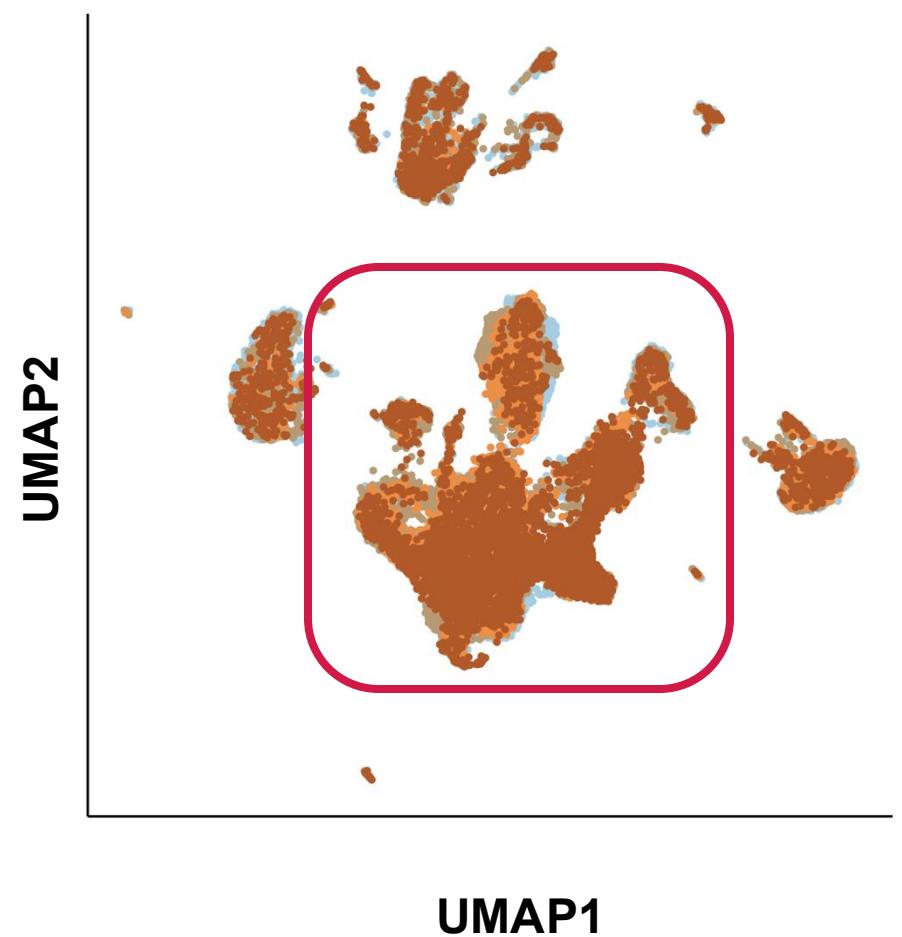
- Many tools, methods for integration
- Bioinformatics core uses three methods
 - Harmony (separate package)
 - LIGER (separate package)
 - Anchor-based integration (implemented in Seurat)
- Harmony attempts to reduce technical variation, maintain biological variation
- LIGER assumes batch effects are technical
- Step-wise anchor-based integration saves resources, can introduce bias
- No consensus on “best” method, depends on size, complexity, etc.



Non-integrated:



Integrated:



UMAP2

UMAP2

UMAP1

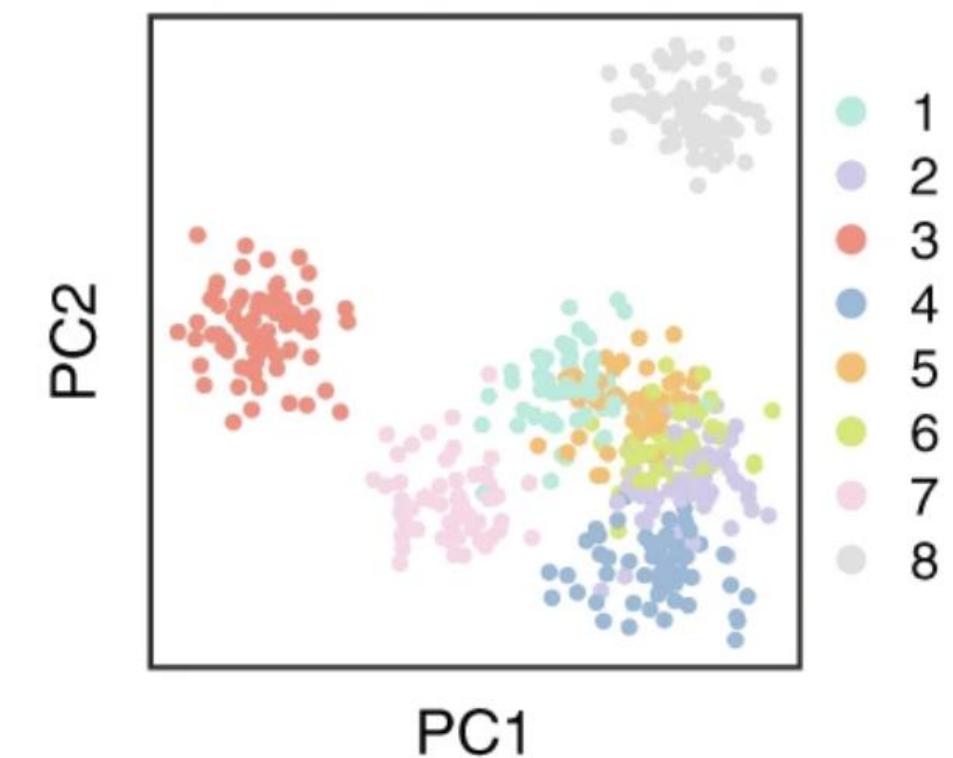
UMAP1



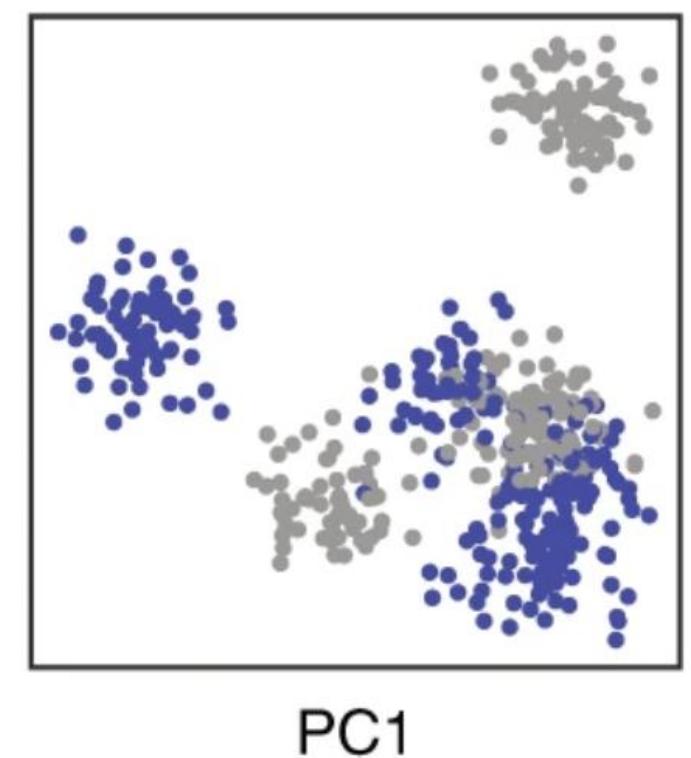
Differential Expression Analysis

- Consider batch effects when running, interpreting DE
 - Technical and biological effects
 - Dropout, inflated zero counts
 - High variability across samples

High variation between replicates



● Control ● Treatment

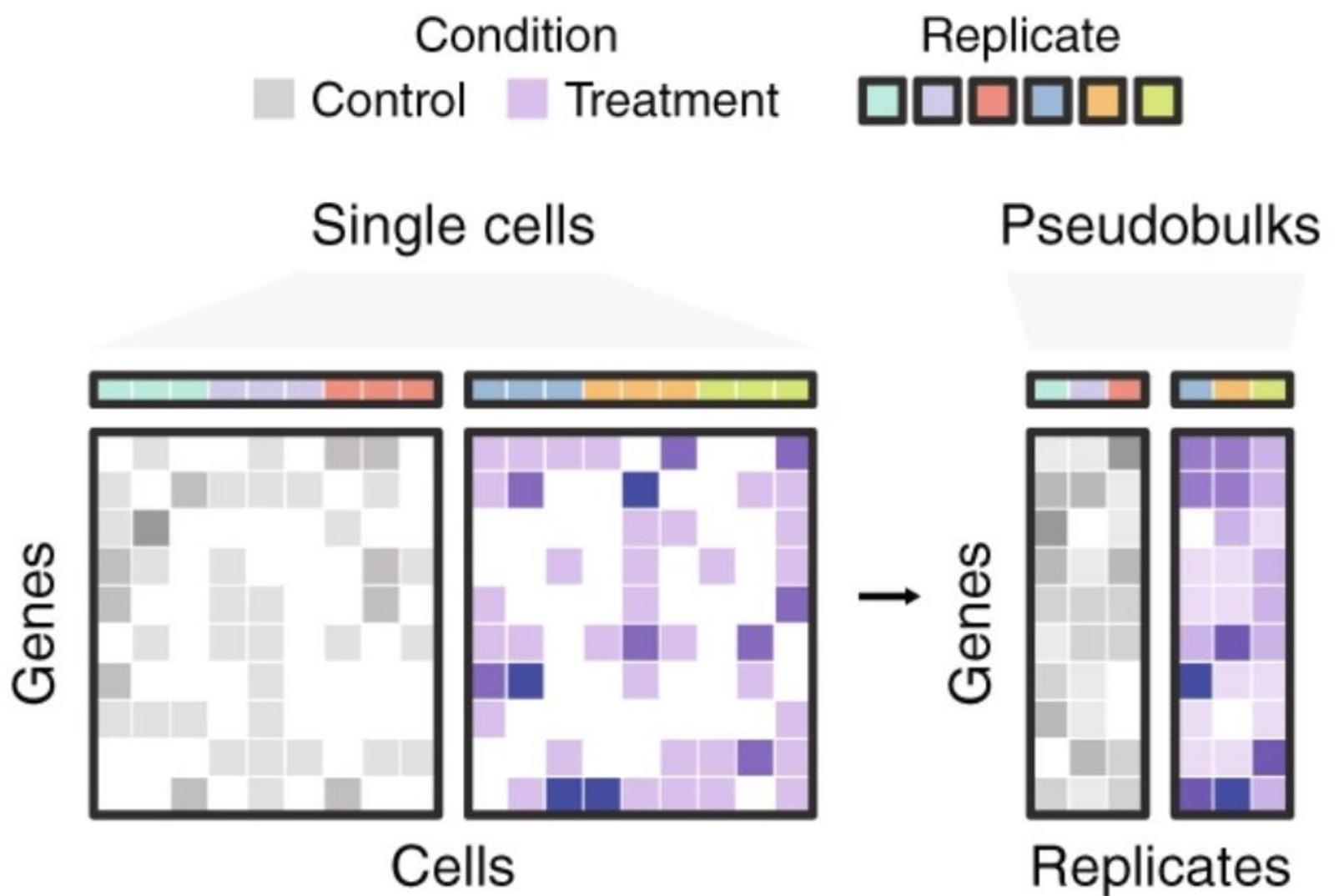


[Squair \(2021\). Nature Communications.](#)



Differential Expression Analysis

- Two categories for methods
 - Cell-level
 - Pseudobulk
- Cell-level treats cell expression profiles individually
 - e.g. Wilcoxon rank sum (Seurat's default)
- Pseudobulk aggregates expression profiles per sample
 - e.g. DESeq2 (bulk RNA-seq method)



[Squair \(2021\). Nature Communications.](#)



Differential Expression Analysis

- Cell-level methods prone certain issues
 - Inflated significance of p-values
 - Inflated number of false positives
- Fail to address dependence of cells in same sample
- Still useful for broad results
 - e.g. generating per cluster markers



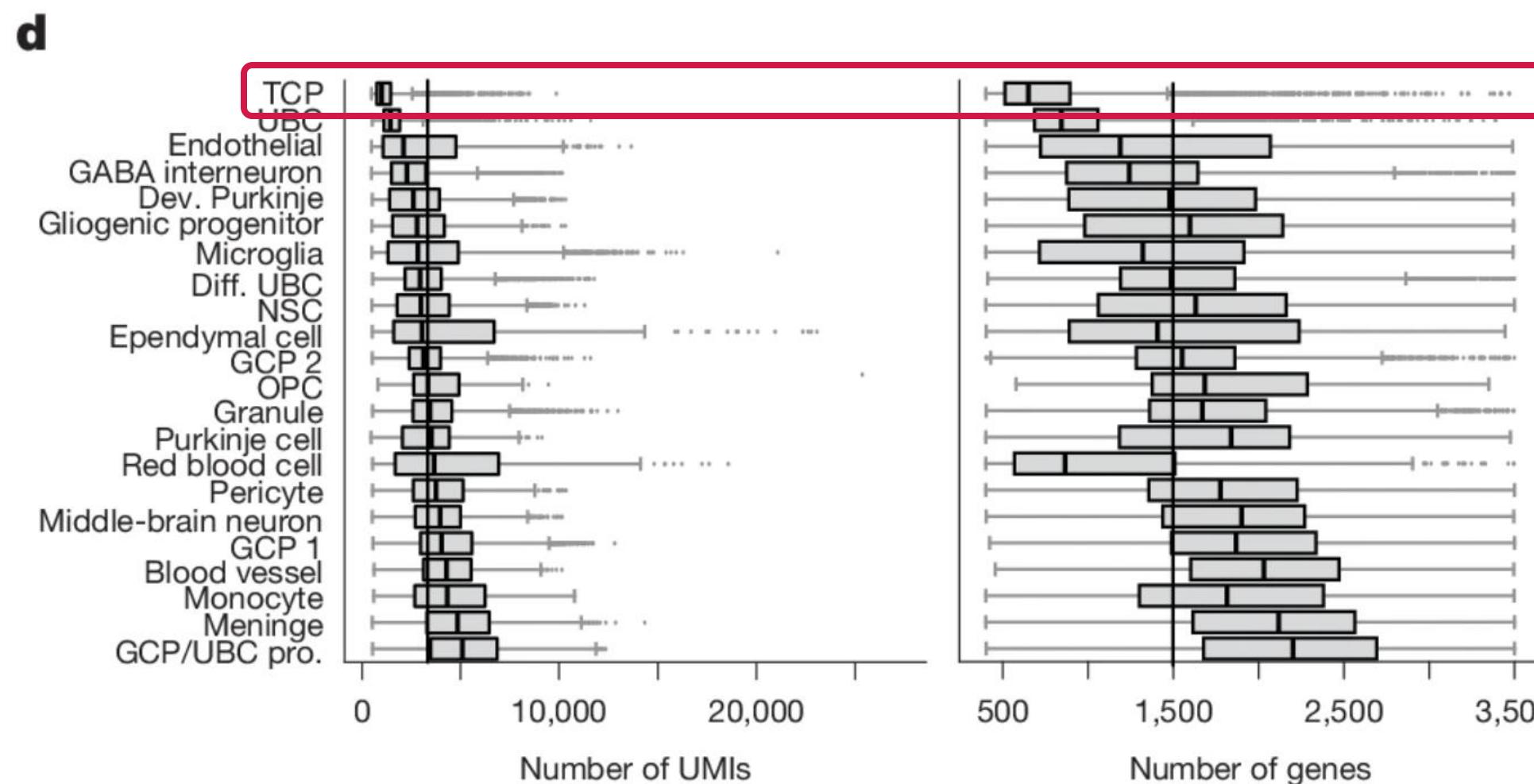
Differential Expression Analysis

- Pseudobulk methods address certain issues
 - Reduce inflated significance
 - Reduce false positive rates
- Allow more complex models, confounding variables
- Need at least two, ideally three replicates per variable of interest
- Can still see batch effects, inflated significance, false positives
- Reproduce results in other datasets
- Confirm via independent wet lab methods



Cell Type Annotation

- Pitfalls upstream can lead to inaccurate annotations downstream
- Inaccurate annotations can lead to false conclusions



[Smith \(2025\). Nature.](#)

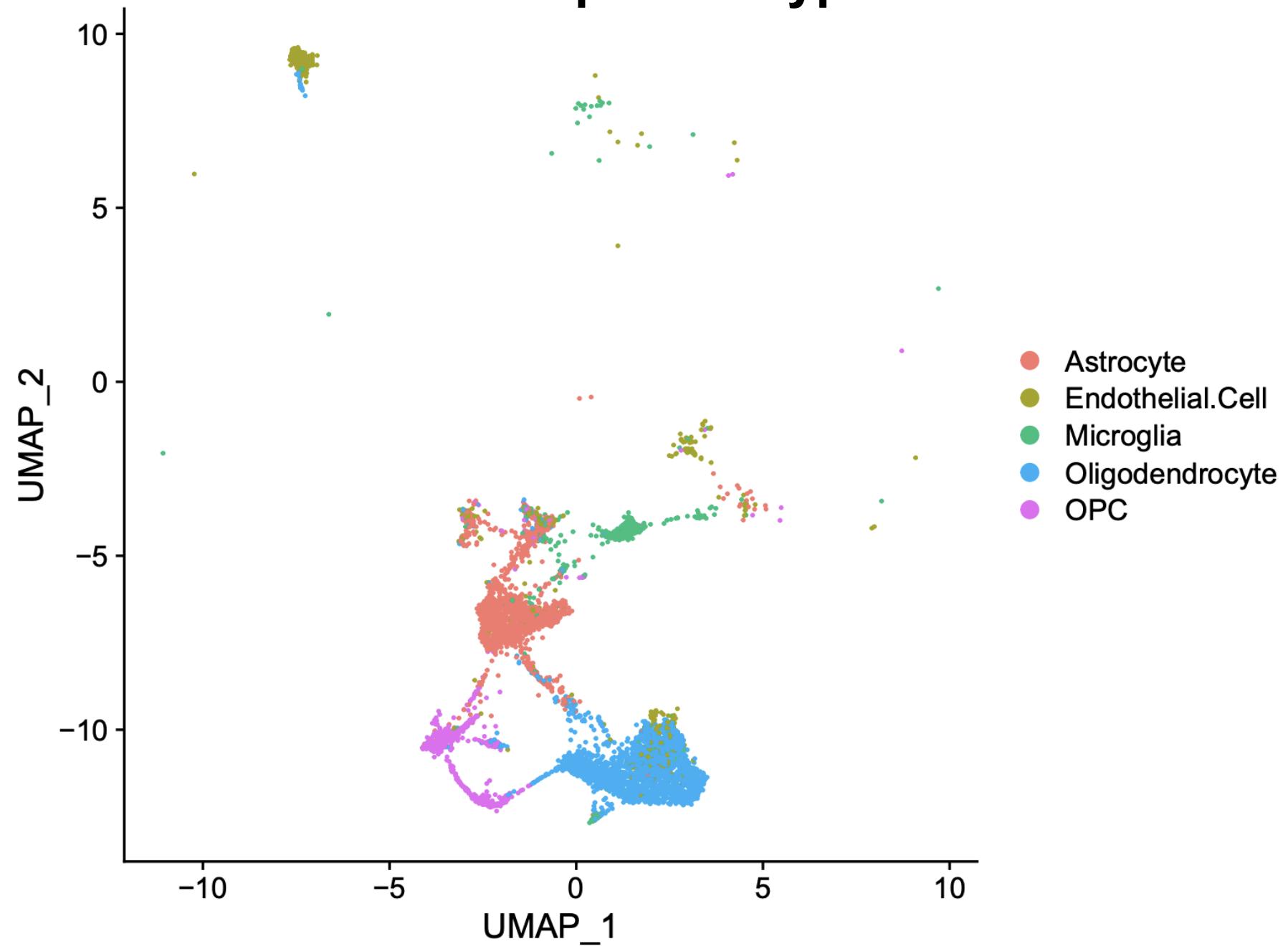


Cell Type Annotation

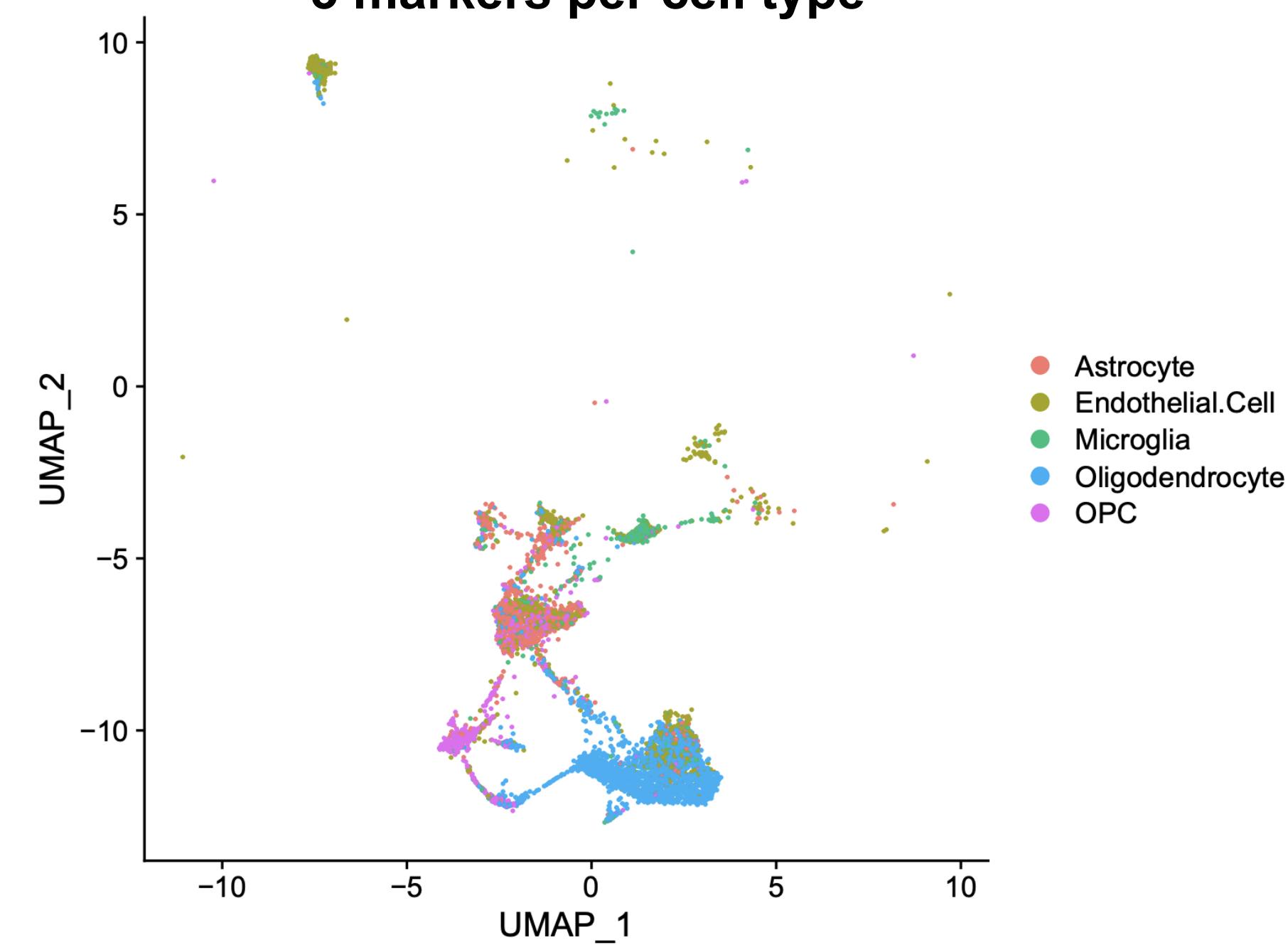
- Annotations can involve manual curation, semi-automated methods, fully automated methods, e.g.
 - Module scoring (semi-automated, uses curated gene marker lists)
 - SingleR annotation (automated, uses annotated single cell or bulk references, generates broad or fine labels)
 - Reference label transfer (automated, uses annotated single cell objects)
- Consider number of cell type markers use for module scoring
 - Too few, overlapping markers can cause mislabeling, miss subtypes
 - More makers reduces noise in annotations
- Module scoring will force label assignment
 - Can only assign labels provided as input



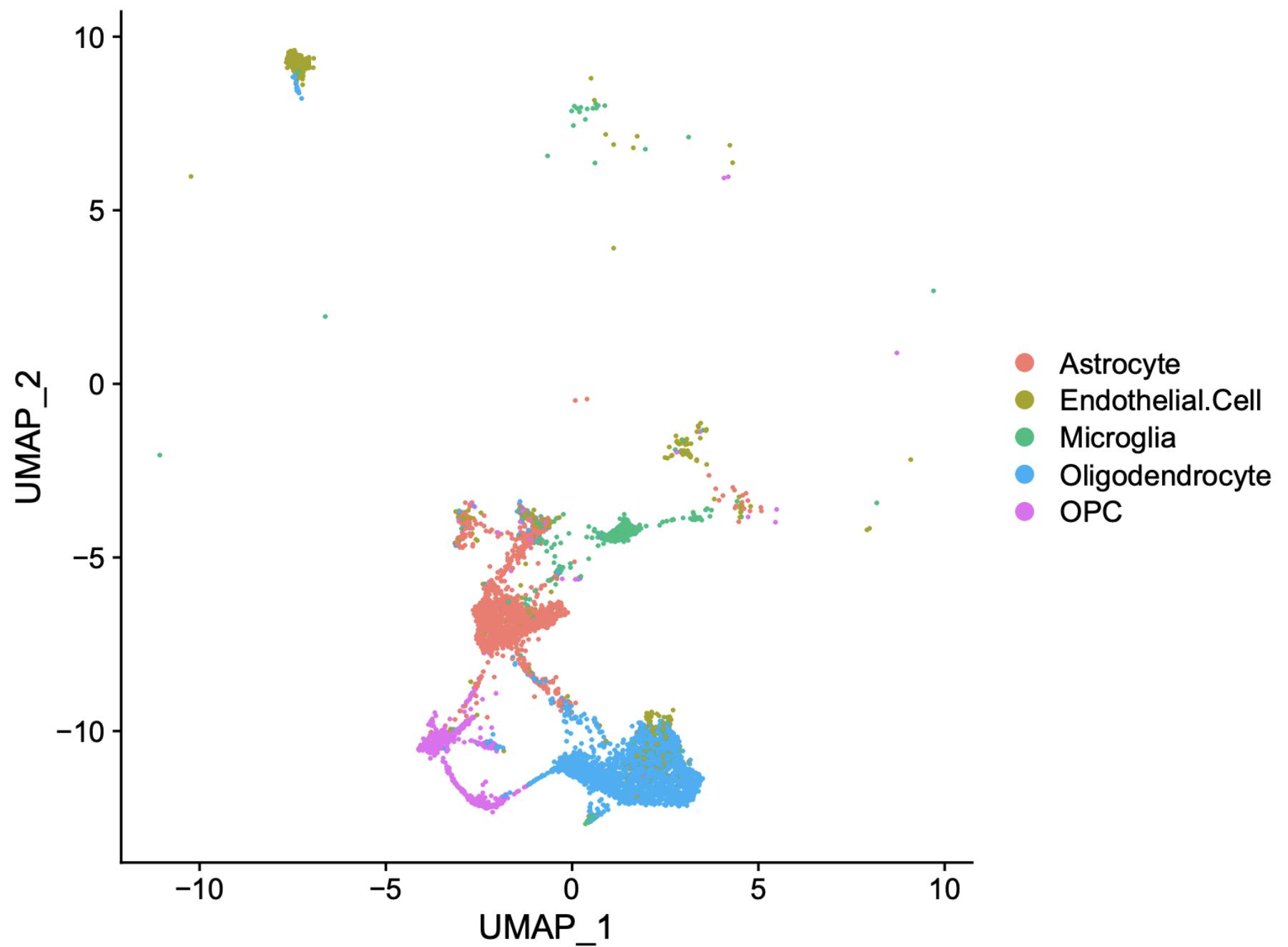
≥ 17 markers per cell type



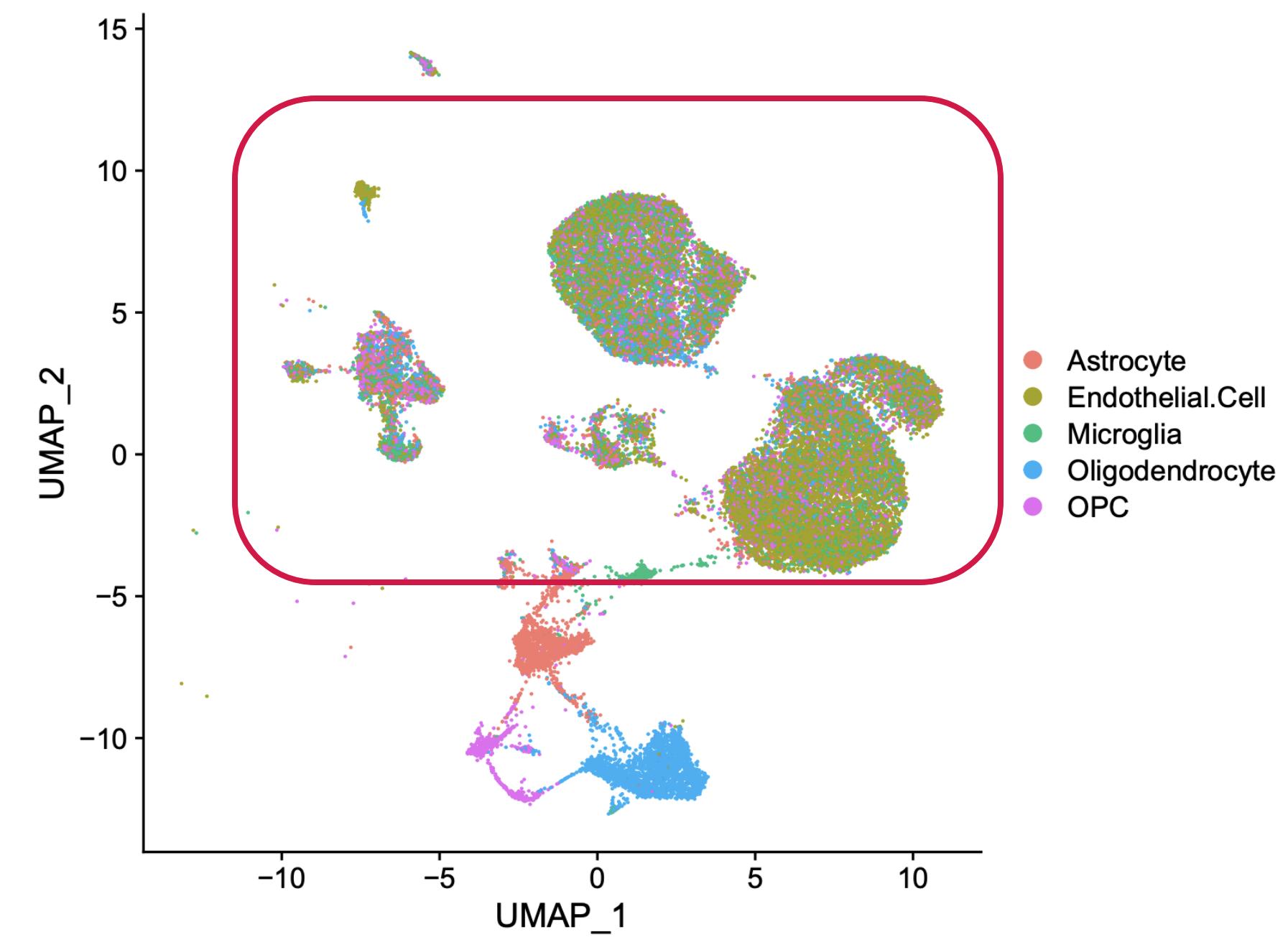
3 markers per cell type



Non-neuronal cells



Neuronal cells

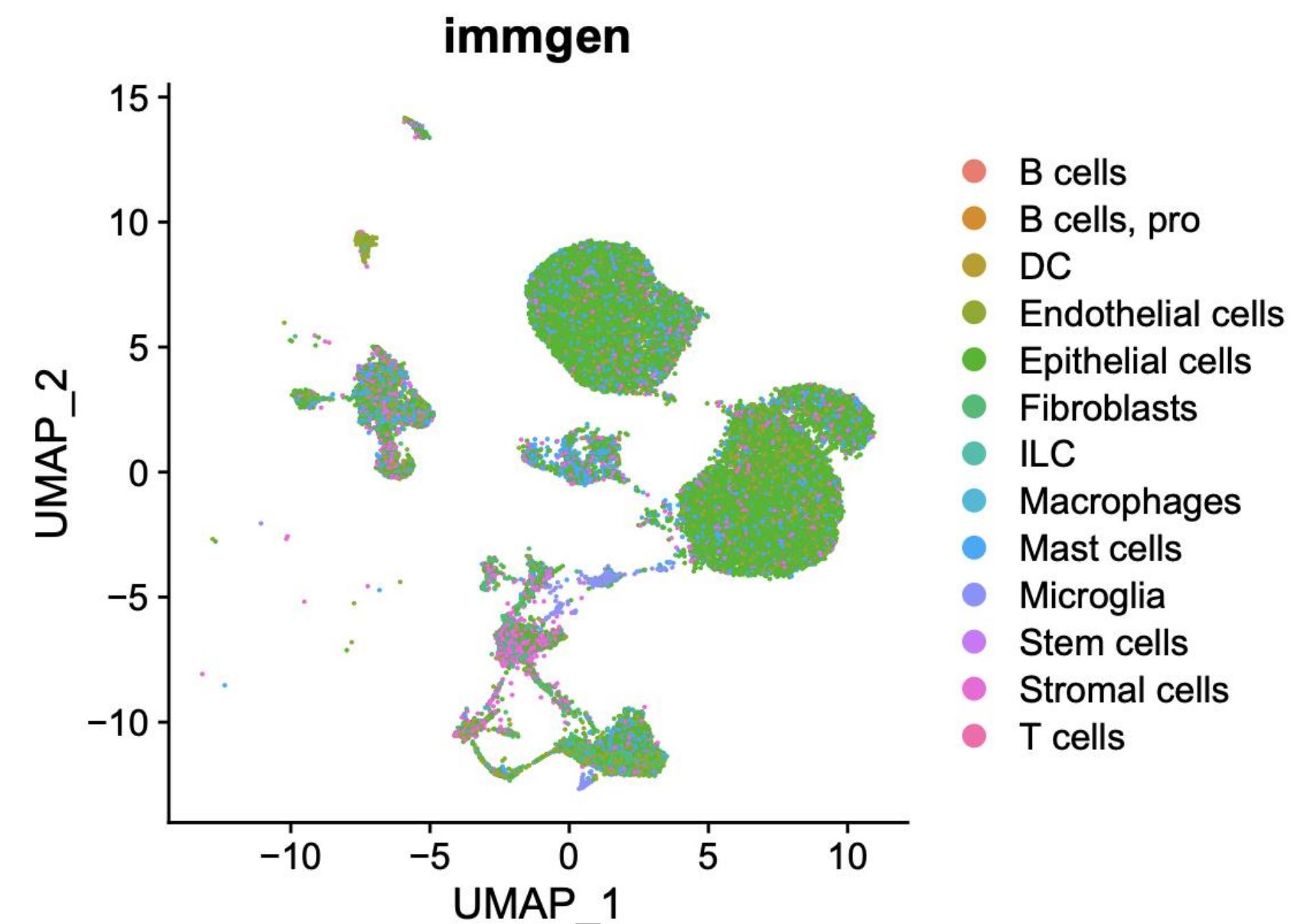
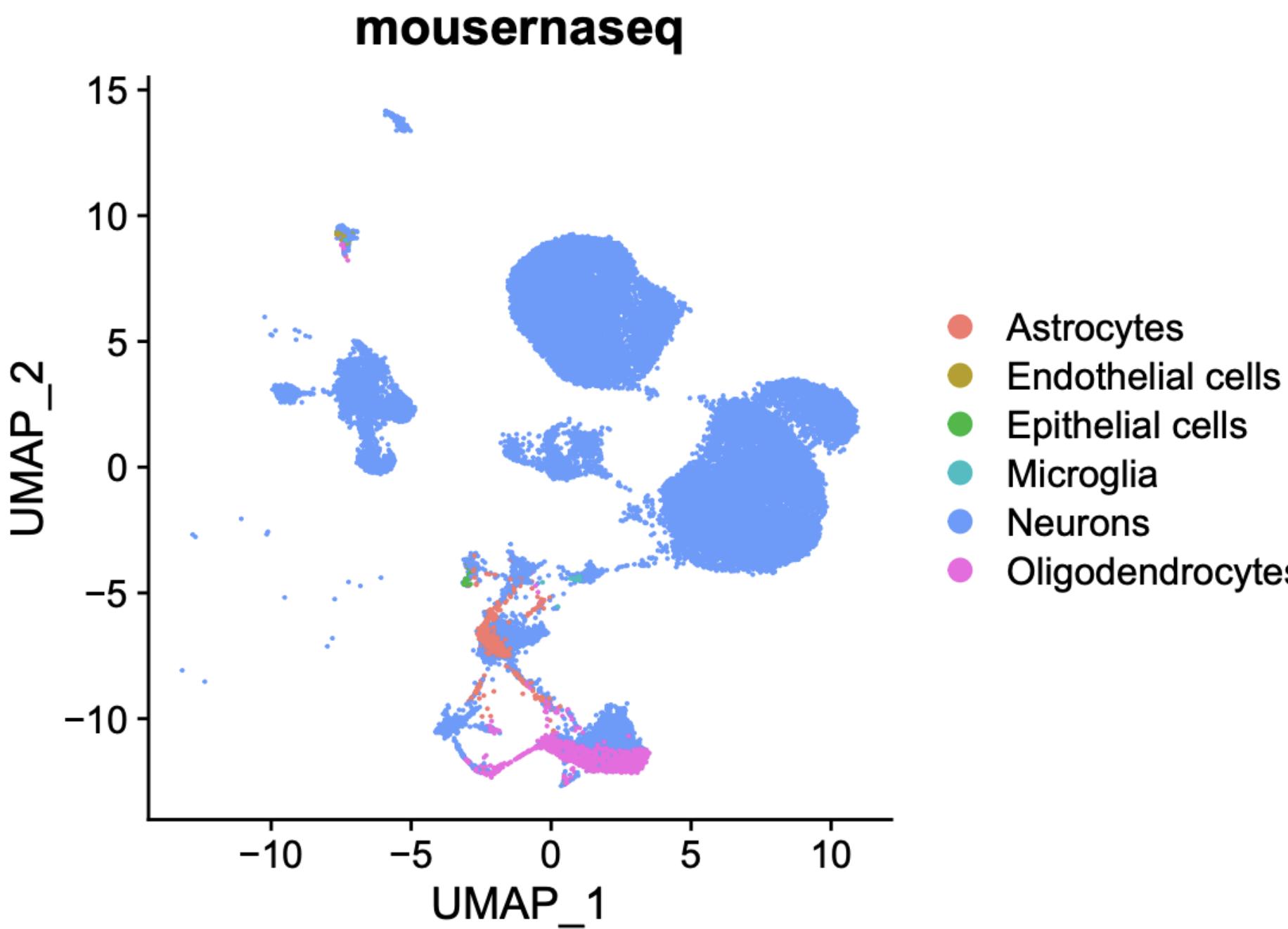


Cell Type Annotation

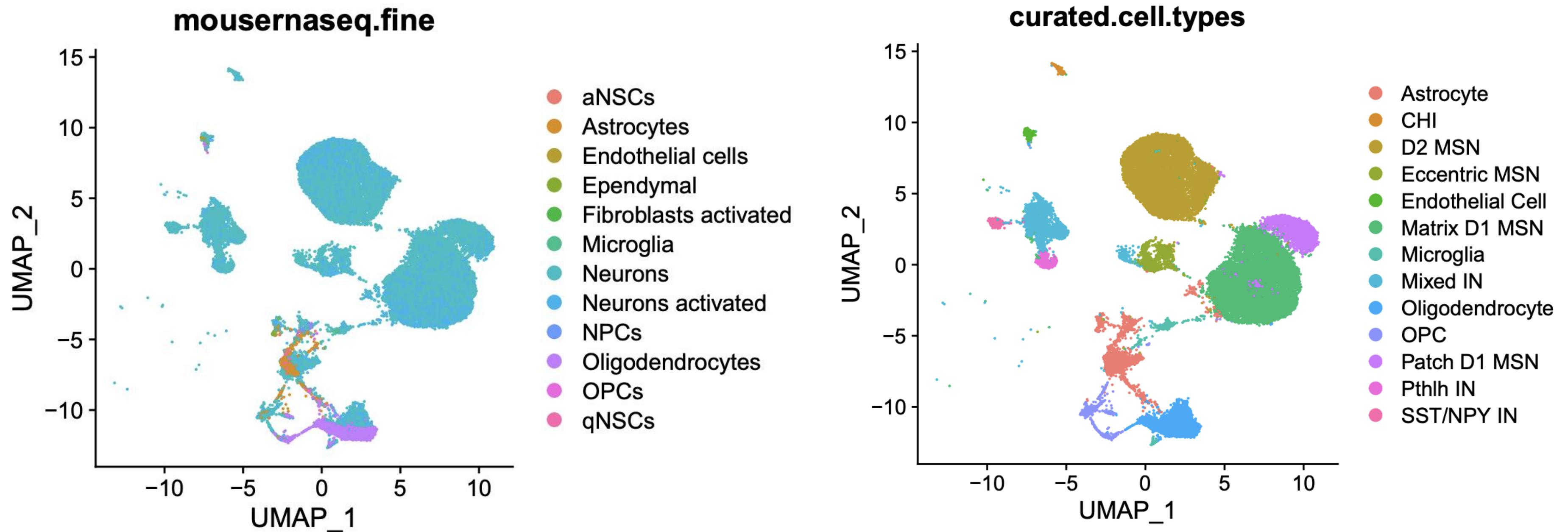
- Important to understand resources used for automated tools (e.g. SingleR, reference label transfer)
 - How are cell types annotated?
 - What cell types are present?
 - How detailed are cell type labels?
- Do methods force labels to be assigned?
- SingleR provides broad, fine labels, can prune cells
- Label transfer detail depends on reference, labels every cell
- Rely on combo of semi-automated methods, fully automated methods, manual annotation



Cell Type Annotation



Cell Type Annotation



Summary

- Pitfalls can occur during most steps
- Analysis often includes redoing, refining steps
- These pitfalls can give you a starting point for fine tuning
- Be careful not to overinterpret data
- Be careful not to overly adjust to fit expected biology
- Verify findings with additional methods
- Gold standard is wet lab verification

